Thesis Title:

"Metabolism of nitrogenous wort components by brewers' yeast"

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ABSTRACT

A quantitative and qualitative identification of the nitrogenous constituents of unfermented and final fermented wort has been performed for five industrial yeast strains. Results highlight the importance of three key amino acids in yeast fermentation performance. Supplementation of wort using individual amino acids and ammonia clearly demonstrate lysine and arginine to be marker nitrogen wort constituents. Synergistic effects of individual wort free amino acids and ammonia on yeast fermentation efficiency were also examined. Further analysis of nitrogeneous wort constituents included examination of oligopeptides with a molecular weight of less than 500 Daltons and the influence of yeast proteases on the availability of small peptides during fermentation. Both lager and ale yeast can simultaneously use amino acids and small peptides as sources of assimilable nitrogen. Laboratory malt and mash analysis of 28 malt types revealed that 80-95% of total free amino nitrogen originates during malting and the remaining 5-20% during mashing.

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TABLE OF CONTENTS

GLOSSARY OF ABBREVIATIONS

ABV: Alcohol By Volume

AHAS: Acetohydroxyacid Synthase

AICAR: 5-Phosphoribosyl-5-Amino-4-Imidazole Carboxamide

ATP: Adenosine Triphosphate

BBMII: Phosphoribosylformimino-5-Aminoimidazolecarboxamide Ribonucleotide

CoA: Coenzyme A

DAHP: Deoxy-D-Arabino-D-Heptulosonate-Phosphate

DO: Dissolved Oxygen

ECD: Electron Capture Detector

FAN: Free Amino Nitrogen

FID: Flame Ionization Detector

g: Centripetal Acceleration

GAP: General Amino Acid Permease

GDH: Glutamate Dehydrogenase

GOGAT: Glutamine 2-Oxoglutarate Amidotransferase

GS: Glutamate Synthase

HEThDP: Hydroxyethyl-Thiaminin Diphosphate

hL: Hectolitre

HPAE: High Performance Anion Exchange

HPLC: High Performance Liquid Chromatography

HWE: Hot Water Extract

ICBD: International Centre for Brewing and Distilling

IPM: a-Isopropylmalate

NADPH: Nicotinamide Adenine Dinucleotide Phosphate

P5C: Pyrroline-5-Carboxylate

PAD: Pulsed Amperometric Detector

PAPS: 3-P-Adenylylsulphate

PFK: Phosphofructose Kinase

PrA: Proteinase A

PR-ATP: Phosphoribosyl-ATP

PRPP: Phosphoribosyl-PP

PTR: Peptide Transport System

PYN: Yeast Peptone Nutrient

rpm: revolutions per minute

S.D.: Standard Deviation

S.G.: Specific Gravity

SCB: Scottish Courage Brewing

TCA cycle: Tricarboxylic Acid

TCA: Trichloroacetic Acid

ThDP: Thiaminin Diphosphate

VDK: Vicinal Diketones

VHM: Very High Maltose

Y_F: Yeast Fraction

YM: Yeast and Mould

PROJECT AIMS

The major focus of this research project was a detailed investigation of the nitrogenous wort components that might play an essential role in brewer's yeast strain fermentation performance. In more detail, a quantitative and qualitative identification and determination of malt nitrogen compounds that affect yeast metabolic activity, in terms of oligopeptides, ammonium salts and both total and individual amino acids, has been conducted. Moreover, the perception of any key "marker" amino acids or other nitrogen malt and wort constituents responsible for stimulating and reinforcing yeast fermentative activity was taken into account. Furthermore, the possibility of any synergistic effects between wort free amino acids, small peptides and ammonia, in terms of improved yeast fermentation efficiency, is a further important attribute that was examined. Finally, any "key" residual amino acids and nitrogen metabolism by-products (for example volatile compounds) were studied in order to determine their relationship with the flavour, character and stability of the finished beer.

Chapter 1: Introduction

1.1 The brewing process; an ancient biotechnology

Beer fermentation is one of the oldest biotechnological processes known to mankind (**Figures 1 & 2**). The basic principle of this procedure is the conversion of a sweet viscous raw material extract known as wort, into an alcoholic product, which is beer. The usual microorganism which gives rise to beer, are strains of brewer's yeast. These yeast strains carry out the metabolism of wort carbohydrates into ethanol and carbon dioxide and at the same time enhances the beer with essential flavour components. The two main types of beer, lager and ale, are produced with yeast strains that belong to the species *Saccharomyces uvarum* (*carlsbergensis*) (now known as *Saccharomyces pastorianus*) and *Saccharomyces cerevisiae*, respectively (Stewart and Russell, 1998). In general terms, it can be said that brewing yeast strains function under a collaborative pattern in which they are supplied with the necessary nutrients that are optimal for their growth and proliferation and simultaneously they produce a refreshing beer with satisfactory quality and stability.

Rapid changes in conventional brewing are currently occurring and there is a trend in the industry to combine the traditional principles of brewing with the technological advances that are available. However, the recipe for making beer by using water, malted barley, hops and yeast still remains the authentic one.

Figures 1 & 2: Egyptians and Babylonians make beer

1.2 Outline of malting and brewing

The whole process commences with malting. Barley grains that have been harvested, stones and unnecessary plant materials removed, are steeped in water or stored in a moisture saturated environment. This steeping induces germination of the barley grains, where the enzymes, α and β amylases are produced in order to break down starch to fermentable sugars. Simultaneously, barley proteases are also activated for the hydrolysis of barley endosperm proteins into smaller proteins, peptides and individual amino acids.

When this germination step is completed, the malted barley is placed in an oven, a process called kilning, in order to halt further endosperm break down and to provide malt with the storage tolerance for a long period of time. The time and the temperature of the kilning determine the beer type that is going to be eventually produced e.g. malt for stout production is highly roasted and turns black. Also this step contributes to the flavour of the final beer.

After the malt drying, the milling stage follows. Malt that has been first cleaned and weighed accurately is placed in a hammer or roller mill in order to be crushed and flour

known as grist, is produced. When malt is ground into powder this is called fine grist and when is ground less thoroughly the flour is called coarse grist. Other cereal adjuncts, e.g maize and rice can also be used for the preparation of the grist that they are usually milled separately from the malted barley. The grist is placed in a mash tun so that the process known as mashing can take place. Cereal adjuncts before being added to the mash tun with the malt grist are first added in a cereal cooker to release fermentable sugars (glucose, fructose, sucrose, maltose and maltotriose). Then they are added to the malt mash and the grist mixture is treated with hot water at temperatures between 55 to 65^{o}C, according to the mashing method being used. During mashing, malt enzymes are re-activated to continue further endosperm degradation for the production of extra fermentable carbohydrates and nitrogenous materials. After completion of protein degradation and saccharification, the mash is raised to a "mash-off" temperature at approximately 75^{o}C and is held at this temperature for a short period to terminate the enzyme activity. At the end of this process ("mash-off" at approximately 75^{o}C), a sweet viscous solution is produced. The aqueous extract, known as wort, is clarified to remove the remaining malt husks by recycling through a lauter tun or a mash filter. The modern mash filter uses a finely hammer-milled grist with a very small particle size compared with that used in a lauter tun and this difference has as a result the reduced bed permeability of the bed produced with the mash filter (Andrews, 2004). Conversely, the bed depth produced with a mash filter is ten times smaller compared to the bed depth formed with a lauter tun and this is sufficient to counteract the loss of flow rate due to the lower bed permeability (Andrews, 2004). The mash filter has higher extract efficiency than the lauter tun, since with the lauter tun it is common to sacrifice a small amount of extract in the last part of the runoff in order to improve cycle times. In addition, lauter tuns can successfully accommodate a load between -50 and +30% of its nominal output, but the mash filter is more restricted and can accommodate only a load between -20 and +10%. Consequently, the mash filter is more effective with a relatively small recipe portfolio and fixed brew size. However, the mash filter loading flexibility can be increased by the use of a dividing plate to reduce the effective size of mash filter. Another difference between these wort separation methods is that the mash filter is particularly good at reducing oxygen pick up (Andrews, 2004). Colloidal haze and settleable solids levels are appreciably better with the mash filter because the filter cloth is the main filtration device and its weave determines the size of particle that can pass through during runoff. By constant, a lauter tun false bottom is not part of the filtration

mechanism and its function is purely to support the grain bed, which is the filter. Another important area of difference, caused by the aggressive hammer-milling of the malt, is the acrospires damage compared with that caused by roller-miller malt for use in a lauter tun and this difference leads to higher polyphenol levels in the wort. Moreover, the spent grain produced by a mash filter is considerably drier than that produced by a lauter tun, typically 70% moisture by weight compared with 78-80%. The process of removing spent grain can be a problem with the mash filter while with modern lauter tuns is more effective, lasting only 10-20min (Andrews, 2004).

The next stage of the process involves the collection of the wort in a canister called either a copper or kettle, hops are added and the mixture is boiled for a specific time for sterilization and also for allowing isomerisation of hops acids. In addition, wort boiling deactivates the wort enzymes, which are still functional and moreover it plays an important role in the coagulation of the mixture's unstable proteins, which produce the trub. At this stage, soluble adjuncts such as glucose and maltose syrups can be added to the kettle in order to provide the fermentation medium with a higher percentage of fermentable sugars (high gravity wort). The wort supplementation with hops in various forms, such as pellets, flowers or extracts serves a binary purpose. Firstly, hops are used as anti-microbial agents in wort, green and finished beer (Shimwell, 1937) and secondly they provide bitter hop aroma and flavour (bitterness) in final beer.

Finally, wort is cooled, aerated or oxygenated, prior to the addition of the yeast (pitching). The wort oxygenation is required prior to yeast pitching because brewing yeasts in the absence of molecular oxygen are unable to synthesize sterols and unsaturated fatty acids, which are essential yeast membrane components and are present in wort in suboptimal quantities (Russell, 1995). Underaeration leads to limited synthesis of essential membrane lipids, which reflects itself in restricted yeast growth, low fermentation performance and many off flavor problems (Russell, 1995). The amount of oxygen required depends mainly on yeast strain, wort gravity and temperature and amount of trub in the wort (Russell, 1995). Wort oxygenation is most commonly conducted by direct injection of either sterile air or oxygen at the time wort exits the wort cooler (Russell, 1995). Pitching of the wort then takes place in the fermentation vessel, where yeast is added to the vessel in a form of a yeast slurry. When fermentation is complete and all the sugars have been converted into ethanol, CO_2 and flavour components, the produced yeast crop can be used for serial repitching of other fermentations for 8 to 10 generations (cycles), according to its viability. The green beer

is stored for maturation in casks or conditioning vessels for 3 to 5 days for ales and up to 3 to 4 weeks and sometimes longer for lagers. The final beer is separated from the residual yeast by filtration or centrifugation and the yeast, protein and polyphenol free beer is stored in kegs, bottles and cans before being distributed in the marketplace for consumption.

1.3 Brewing yeast growth and fermentation performance

Yeast growth is concerned essentially with how yeast transports and assimilates nutrients in the cell in order to increase in mass and eventually divide (Walker, 1998). Fermentation performance is defined as the capacity of a brewer's yeast to consistently exhibit four basic key attributes: **1)** cell proliferation, **2)** utilization of the available carbohydrates in order that ethanol (and other by-products) production occurs (attenuation), **3)** the accumulation and subsequent sedimentation of yeast biomass at the end of fermentation process (flocculation) and **4)** desirable flavour development (Smart, 2000). Brewing yeast's metabolic activity is strongly affected by its physiological condition. This means that brewers require consistent yeast quality and quantity.

1.4 Brewing yeast nutrition

After pitching, the yeast starter culture must adapt to the highly complex wort environment (Stewart and Russell, 1998). It is known that wort is a yeast growth medium that consists of sugars (fructose, sucrose, glucose, maltose, maltotriose, and non-fermentable dextrins), nitrogenous materials (amino acids, peptides, proteins, nucleic acids, malt endosperm degradation products), vitamins, ions, mineral salts, trace elements, fatty acids and so many other chemical constituents. The fermentable carbohydrates supply the yeast cells with energy in the form of ATP and also carbon skeletons for biosynthetic purposes (Hough *et al*. 1982). Amino acids, on the other hand, are utilized by the yeast as the "building blocks" for the biosynthesis of newly formed proteins, both enzymatic and structural, and new amino acids (*de novo*) (Hough *et al*. 1982). The rest of the above mentioned wort constituents are essential for the membrane synthesis of the daughter yeast cells.

1.4.1 Carbon

As previously mentioned, brewer's wort contains a number of sugars. The most abundant sugar in wort is maltose, which constitutes 50 to 60% (w/v) of the total wort sugars, followed by maltotriose, which constitutes the 15 to 20% (w/v) of total wort carbohydrates. Glucose and fructose are the main monosaccharides in wort and they comprise the 9-10% of the total wort carbohydrates (Hornsey, 1999). Dextrins are also wort carbohydrates that cannot be metabolized by the yeast and as a result they remain in the final beer contributing to its flavor, body (mouth feel) and calories. The main function of wort sugars is their conversion by yeast into ethanol and carbon dioxide so that energy is provided for yeast proliferation and maintenance of cell viability and vitality. Simultaneous production of higher alcohols, esters and other flavour compounds is induced by sugar metabolism, which are key compounds for beer flavour.

1.4.2 Oxygen

Brewing fermentation is essentially an anaerobic process, but when wort is inoculated with yeast some dissolved oxygen must be available in the medium. However, oxygen present in the later stages of fermentation is undesirable since, it negatively affects the spectrum of flavour compounds in final beer.

Oxygen in wort during the early stages of fermentation is beneficial for yeast cells since it is involved in the synthesis of unsaturated fatty acids and sterols, which are the main and necessary components of cell membranes. Thus, trace amounts of dissolved oxygen in wort have a stimulatory effect upon yeast reproduction and enhance attenuation, high gravity brewing fermentation rates and prevent undesirable odors in beer (Kaneda *et al.* 1992). However, the oxygen present at yeast pitching has been completely utilized during the initial ten hours of fermentation.

1.4.3 Inorganic ions

Brewing yeasts also need a wide range of inorganic ions for efficient proliferation and fermentation performance (Walker, 2000). The major function of these wort components is both structural and enzymatic. Changes in the levels of these ionic forms result in alteration of yeast metabolism and growth profile, for instance the morphology of the

cells is influenced (Walker, 2000). A number of these ions function as the catalytic center of an enzyme in a metabolic pathway, as an activator or stabilizer of an enzyme function and maintains physiological control by competition between activator and deactivator. Zn^{+2}, Co^{+2}, Mn^{+2} and Cu^{+2} are catalytic centers while Mg^{+2}, acts as an enzyme activator.

Concerning their function in enzyme structure, ions act to neutralize forces present in various cellular anionic units. For nucleic acids and proteins, Mg^{+2} and K^{+} are most commonly encountered in this role (Walker, 2000). Calcium ions are involved in the yeast cell membrane structure and function. Calcium also plays an important role in yeast flocculation.

1.4.4 Vitamins

Wort is also a vitamin rich medium, containing biotin, thiamine, nicotinic acid, fiboflavin, inositol and many others. Almost all of these vitamins, except mesoinositol, are required by yeast in order to activate coenzymes. Lack of vitamins in the fermentation medium, especially biotin and inositol deficiency, leads to many fermentation problems due to malfunction of yeast metabolic activities.

1.4.5 Nitrogen

Nitrogen is also an essential element for yeast growth and function. Active yeast growth involves the uptake of nitrogen and as the yeast multiplication stops, the nitrogen utilization also ceases or decelerates. However, not all nitrogen materials in the wort can be utilized by the yeast in order to carry out its metabolic activities. The nitrogenous compounds available for consumption by the yeast are known as assimilable nitrogen or free amino nitrogen.

1.5 Free amino nitrogen (FAN)

FAN (free amino nitrogen) can be defined as the sum of the individual wort amino acids, ammonium ions and small peptides (di-, tripeptides). FAN is believed to be an important general measure of those yeast nutrients which constitute the yeast assimable nitrogen in brewery fermentations (Pugh *et al.* 1997). It is common knowledge that even if

attenuation of wort carbohydrates proceeds normally, the same quality of beer is not always guaranteed to be produced, suggesting that the sugar content of wort alone is not a good indicator for yeast fermentation performance (Inoue and Kashihara, 1995). Thus, FAN has been regarded as a better index for prediction of healthy yeast growth, viability, vitality, fermentation efficiency and hence beer quality and stability. In addition, the nitrogen obtained from wort is used by the yeast to accomplish its metabolic activities, which are the synthesis of amino acids (*de novo*) and ultimately structural and enzymatic proteins intracellularly (Taylor and Boyd, 1986). The majority of these wort nitrogenous components are consumed within the first 24h of incubation until active yeast growth stops (Ingledew, 1975). However, there are also differences between lager and ale yeast strains with respect to wort assimilable nitrogen uptake characteristics (Ingledew, 1975).

Adequate levels of FAN in wort ensure efficient yeast cell growth and hence desirable fermentation performance. The amount of wort FAN content required by yeast under normal brewery fermentations is directly proportional to yeast growth and also affects beer maturation (Pugh *et al.* 1997). There is also a strong correlation between the initial FAN levels and the final level of ethanol produced (Pickerell, 1986). It is therefore of considerable importance to ascertain that the FAN levels of the wort are high enough to support a rapid and normal fermentation (Pickerell, 1986). The minimal FAN level to achieve satisfactory yeast growth and fermentation performance in normal gravity fermentations is 130mg/L, but for rapid attenuation resulting in higher ethanol production, increased levels of FAN are required (170-190mg/L) (Jones and Ingledew, 1994). Meilgaard (1976) suggested that in normal wort gravity fermentations, a minimum level of 150mg/L FAN is required to permit rapid and complete attenuation. However, optimal FAN levels differ from fermentation to fermentation and from yeast to yeast, thus they are considered as controversial and unverified. Furthermore, the optimum FAN values change with different wort sugar levels (Pickerell, 1986). While FAN concentration in malt provides adequate information for nitrogen nutrient availability, total nitrogen and the permanently soluble nitrogen are perhaps better indices for beer foam stability (Jin *et al*. 1996).

FAN is the protein degradation product of various raw materials, such as malted barley, wheat, sorghum etc. (Taylor and Boyd, 1986) and it is affected by the malt/adjunct ratio, mashing schedule, barley variety and malting conditions (Pugh *et al.* 1997). The greater part of FAN (70%) found in wort is pre-formed in malt at low temperatures, whilst the remaining part of nitrogenous materials (30%), originates from the action of proteases

during mashing (Lie, 1973). Since large proportions of starchy adjunct are used in mashing, as a less expensive way of producing a yeast growth fermentation medium (Casey *et al.* 1984), the FAN content of the medium becomes so low to prematurely terminate cell division leading to prolonged and in many cases to sluggish and incomplete fermentations (Pickerell, 1986 and Casey *et al.* 1984). Thus, it is vital to have extensive proteolysis in adjunct worts during mashing to ensure adequate levels of wort assimilable yeast nitrogen (Ingledew, 1975).

In addition, it appears that *Saccharomyces cerevisiae* under conditions of high gravity brewing, needs extra FAN to cope with osmotic stress and other stress conditions (Thomas *et al.* 1996). This means that as wort gravity increases, the levels of assimilable nitrogen should also increase in order that a certain rate of glycolytic flux and high cell viability is maintained (Thomas *et al.* 1996). It is important that with increases in the percentage of dissolved sugars in the wort, a simultaneous increase in assimilable nitrogen also takes place, so that the brewer creates larger economies in labour, capital expenditure, energy and higher ethanol production and avoids stuck fermentations and yeast crops, which due to their low viability, cannot be repitched (Casey *et al.* 1984).

Jones and Pierce (1964) found that the absorption and utilization of exogenous nitrogenous wort compounds and their synthesis intracellularly are controlled by three main factors: a) the total wort concentration of assimilable nitrogen, b) the concentration of individual nitrogenous compounds and their nature and c) the competitive inhibition of the uptake of these components via various permease systems.

Finally, FAN is not only used to provide nitrogen to the yeast cells for growth but also the wort nitrogen content or its metabolic products affects the flavour and stability of beer (Pickerell, 1986).

1.5.1 FAN production during malting

One important aspect of barley and malt studies is the relationship between the level of nitrogen incorporated into barley during germination and growth in the field and the level of enzyme activities developed in barley during malting (Agu, 2003). For a normal brewing process, the relative composition of individual amino acids is relatively constant whereas the total FAN varies considerably (Lie, 1973). Determination of the total FAN in malt and wort provides sufficient information for quality control purposes unless there are major changes in the raw materials or in the brewing process employed (Lie, 1973).

Proteolysis is important during malting because the soluble nitrogen pool required for enzyme synthesis is produced when proteolysis is optimal (Bishop, 1928; Palmer, 1989, Pollock *et al*. 1959). This is greater when most of the soluble nitrogen pool is retained within the grain rather than transferred in the roots and shoots (Agu, 2003; Taylor, 1983). The nitrogen content of barley affects the extent of hydrolysis and hence endosperm modification achieved during malting (Palmer, 1997; 1999).

During malting the storage proteins of the grain endosperm are hydrolyzed by proteases into polypeptides, oligopeptides and amino acids (Agu and Palmer, 1999). Germinating barley contains a large number of peptide hydrolases including carboxypeptidases (Virusi *et al*. 1969; Mikola and Kolehmainen, 1972), amino peptidases (Burger *et al*. 1970) and di- and tripeptidases (Virusi *et al*. 1969; Sopanen, 1976). Endopeptidases are mainly located in the endosperm and the function they serve is to "attack" storage proteins and proteins associated with cell walls and starch granules, thereby exposing the cell wall and starch to amylolytic action (Palmer and Bathgate, 1976). The carboxypeptidases are exopeptidases acting from the carboxyl end of the peptide chain and they are active during germination mainly in the starchy endosperm, where the main storage protein hordein is located (Mikola and Kolehmainen, 1972). These exopeptidases, in conjunction with endopeptidases, are the most important peptidases for hordein hydrolysis during the germination of the grain (Palmer and Shirakashi, 1994; Baxter, 1978). The hydrolysis products of these peptidases are individual amino acids and simple and complex peptides (Mikola *et al*. 1971).

The endosperm proteins that are degraded during malting are most likely a mixture of hordeins (prolamin proteins) and to a lesser extent glutelin proteins (Rastogi and Oaks, 1986; Skerritt, 1988; Weiss *et al*. 1992). It is also possible that hordeins are more completely degraded to amino acids and oligopeptides compared to glutelins, which appear to be a source of larger peptides (Osman *et al*. 2001, 2002). Fujimaki *et al.* (1977) also suggested that prolamins are degraded to low molecular weight peptides without the formation of intermediate polypeptides. The vast majority of prolamin proteins are degraded during malting because the protein which remains is identical to the prolamins from the unmalted grains (Taylor, 1983).

The glutelin proteins were found to constitute the 27% of the total nitrogen of unmalted grains (Taylor, 1983). However, only half of this percentage is degraded during malting to provide assimilable nitrogen for yeast metabolism (Taylor, 1983). This proves the fact that glutelins are quantitatively much less important than the prolamins as storage

proteins (Taylor, 1983). In addition, the initial levels of the albumin and globulin proteins during germination, declined to some 55% (Taylor, 1983). The conversion of these proteins into more utilizable nitrogen forms suggests that they also contribute to a lesser degree in the production of FAN in the malt.

During seven days of malting, the FAN content of the grains increases more than nine-fold, as storage proteins are degraded into amino acids and oligopeptides (Taylor, 1983). Lysine, the first limiting amino acid of cereals, increases four-fold during malting (Taylor, 1983). The proline concentration in wort depends mainly on the proline content of the grist, with only a relatively small amount being produced during mashing (Jones and Pierce, 1964). Changes in the germination temperatures may be beneficial for a greater break-down of the whole protein spectrum during malting (Agu and Palmer, 1999).

1.5.2 FAN production during mashing

In brewing, a period of the mashing cycle is usually carried out at the optimum temperature for protease activity in order for FAN synthesis to be enhanced (Taylor and Boyd, 1986). This period of protein rest is in the temperature range 45-55^{o}C, which is probably optimum for the heat-labile enzymes, the rate limiting enzymes of FAN release during mashing (Sopanen *et al*. 1980). Grain nitrogenous compounds are mainly solubilized during mashing and knowledge of the relative contribution of this grain process step to wort nitrogen is important in indicating whether control can best be exerted in the mashing stage (Barrett and Kirsop, 1971). Wort FAN can be increased by a number of methods during mashing: increasing the ratio of malt to adjunct, adjusting the mashing temperature to the optimum range, adjusting the pH range and by the use of microbial proteolytic enzymes (Taylor and Boyd, 1986).

1.5.3 FAN in wheat

The amount of FAN present in wheat mash is too low to promote yeast growth and fermentation at the optimum rate (Thomas and Ingledew, 1990; Thomas and Ingledew, 1992). A 20^{o}Plato wheat mash has a FAN content of only 70-80 mg/L, very low compared to an all malt barley wort type of equal solids concentration, which is 180-210 mg/L FAN (Thomas and Ingledew, 1992). This is because wheat mash is rich in proteins

that yeast cannot use for growth unless they are first hydrolyzed to simple amino acids and small assimilable peptides (Thomas and Ingledew, 1990). Assimilable nitrogen during wheat mash fermentations is provided from amino acids and to a limited extent from small peptides (Thomas and Ingledew, 1992). The most abundant amino acid in wheat mash is asparagine (Thomas and Ingledew, 1992). However, the fact that even under very severe nitrogen deficiency (58 mg/L FAN), complete attenuation is possible, suggests that the FAN content of wheat mash is qualitatively different from that of wort (Thomas and Ingledew, 1990).

1.5.4 FAN in sorghum

Sorghum malt is notable for high FAN content compared to malted barley (Ratnavathi *et al*. 2000). When sorghum is malted, much of the nitrogen in the kernel travels to the roots and shoots (Taylor, 1983). As in barley malt, prolamins are the main source of the nitrogen transferred (Taylor, 1983). Quantitatively, the major amino acids found in sorghum malt are asparagine and glutamine, in contrast with barley malt where proline is the most abundant amino acid (Taylor, 1983). The proline content of sorghum compared to that of barley is very low (Taylor, 1983). This great difference is possibly related to the fact that sorghum prolamins contain a much lower percentage of proline than the barley hordeins (Taylor, 1983). The fact that γ–amino butyrate is also present in the malt after malting, indicates that FAN does not simply result only from the break down of storage proteins. As γ–amino butyrate is a non protein amino acid degradation product at least some transamination must have taken place (Taylor, 1983). This improvement in the nutritional quality and quantity of FAN mirrors the changes taking place in the total amino acid composition of sorghum during germination (Taylor, 1983).

When sorghum and barley of similar total nitrogen are malted, similar endopeptidase activities were observed (Agu and Palmer, 1999). However, it was found that the solubilities of proteins in sorghum is lower than barley during malting suggesting that the storage proteins of barley and sorghum may be structurally different and/or that the affinities of the barley peptidases may be wider than those of sorghum (Agu and Palmer, 1999). Hence, malted barley produces more FAN than sorghum in their extracts (Agu and Palmer, 1999).

1.6 Amino acids

Amino acids constitute an important fraction of brewer's wort and their determination is therefore of considerable interest in experimental work and in industrial production (Lie, 1973). During fermentation, the amino acids are taken up by the yeast providing the cells with nitrogen (Lie, 1973). The carbon skeletons of the absorbed amino acids are utilized for the biosynthesis of new yeast amino acids and proteins or they may be transformed into flavour compounds (Lie, 1973). The amino acid composition of wort is consequently a rather important part of the complex system regulating the production of flavour active compounds formed by the yeast (Lie, 1973). Brewer's wort contains 19 of the essential 20 amino acids (cysteine is absent), and as with wort sugars, the uptake of amino acids is ordered (Jones and Pierce, 1964).

Jones and Pierce (1964) established a unique classification of amino acids according to their rates of consumption during brewing fermentations (**Table 1**). This categorization is the basis of today's understanding of the relative importance of individual wort amino acids during fermentation and manipulating wort nitrogen levels by the addition of yeast extract or specific amino acids in high gravity brewing (O'Connor & Ingledew, 1989). However, it has been proved that this assimilation pattern of FAN is often specific to the conditions employed and among them the yeast strain's nutritional preferences is perhaps more significant (Jin *et al*. 1996). Thus, because of the differences in malting barley varieties, brewing conditions and yeast strains used in the brewing industry worldwide, a further more detailed review is desirable (Jin *et al*. 1996). Over the years, this amino acid consumption pattern has been confirmed by a number of brewing research laboratories (Jin *et al*. 1996).

Table 1: Classification of wort amino acids according to their consumption rate by an ale yeast (Source: Jones and Pierce, 1969)

Group A Fast Absorption	**Group B** Intermediate Absorption	**Group C** Slow Absorption	**Group D** Little or No Absorption
Glutamic acid Aspartic acid Asparagine Glutamine Serine Threonine Lysine Arginine	Valine Methionine Leucine Isoleucine Histidine	Glycine Phenylalanine Tyrosine Tryptophan Alanine Ammonia	Proline

Amino acids in group A are utilized immediately following yeast pitching, whereas those in group B are assimilated more slowly. Utilisation of group C amino acids commences when group A types are fully assimilated. Proline, the sole group D amino acid, is utilized poorly or not at all during a typical brewing fermentation. Proline is the most abundant amino acid in the wort and is reported as a group D amino acid (Jin *et al*. 1996). However, very little is known about its significance in fermentation, although complete proline utilization does occur under aerobic conditions (Jones and Pierce, 1969; Ingledew *et al*. 1987). Brewer's yeast is able to assimilate proline under aerobic fermentation conditions as a result of the activation of the mitochondrial yeast enzyme proline oxidase, which is responsible for the oxidation of proline to Δ^1-pyrroline-5-carboxylate (P5C) and then P5C is converted to glutamate by the action of P5C dehydrogenase (Ingledew *et al*. 1987).

Yeast cells under conditions of insufficient amino acids of the groups A and B, apparently compensate this situation by carrying out complete utilization of the group C amino acids (Jin *et al*. 1996). Results of several fermentations indicated that this nitrogen compensation mechanism operates only when there is a general shortage of assimilable nitrogen (Jin *et al*. 1996). Differences in the fermentation performance between different fermentations may not be caused by the lack of any particular amino acid group, but by the efficiency of nitrogen utilization (Jin *et al*. 1996). It should also be mentioned that both glycolytic and hexose monophosphate pathways are regulated by nitrogen limitation (Thomas *et al*. 1996). Synthesis of enzymes is affected by changing yeast assimilable

nitrogen from growth limiting to growth non-limiting concentrations. Under nitrogen limitation, synthesis of phosphofructokinase (PFK) by the yeast was found to be 2.5 times greater than when nitrogen was in excess in the medium containing glucose (5% (w/v)) (Thomas *et al*. 1996). Similarly, the amount of hexokinase is 1.6 times higher under nitrogen limitation conditions than under excess conditions (Thomas *et al*. 1996). Therefore, results from enzyme production suggested that the fermentative capacity of yeast (g of sugar consumed per yeast per hour) is higher under nitrogen limitation than that observed under nitrogen excess conditions (Thomas *et al*. 1993). The fermentative yeast capacity varies with the type of nutrient limitation (Thomas *et al*. 1996). In addition, Casey *et al.* (1984) reported that under nitrogen-limiting conditions, the fermentative power of yeast growing in nitrogen deficient wort was equal or even higher than that achieved when the wort was rich in assimilable nitrogen, confirming the above results.

Amino acid uptake decreases with simultaneous increase of the original wort gravity (Takahashi *et al*. 1997). Readily used amino acids such as lysine and methionine are not influenced by the increase in the amount of dissolved solids in wort or the changes in the fermentation temperature or even by changing the yeast strain (Takahashi *et al*. 1997). However, the Group B and C amino acids are strongly affected by changes in the above mentioned fermentation parameters and their uptake rate decreases significantly resulting in high concentrations of these amino acids found in final beer (Takahashi *et al*. 1997).

Intact assimilation of exogenous amino acids into yeast proteins does not occur, they firstly undergo transamination (Ingledew, 1975; Pierce, 1987). The total FAN uptake is led more by the total assimilable nitrogen levels in the wort than by the concentration of specific amino acids (Pierce, 1987). In addition, the order of consumption of wort amino acids is not dependent on the variations of the composition of any single amino acid. Thus, it is clear that for certain amino acids, it is essential that their relative proportions be maintained in wort at standard values whilst, for other amino acids the relative concentrations are not significant apart from their contribution to the total assimilable nitrogen (Pierce, 1987). Amino acids can also be classified according to the essential nature of their keto analogues during yeast metabolism (**Table 2**).

Table 2: Classification of amino acids according to their essential nature (Source: Pierce, 1987)

Class 1	Class 2	Class 3
Aspartic acid	Isoleucine	Lysine
Asparagine	Valine	Histidine
Glutamic acid	Phenylalanine	Arginine
Threonine	Glycine	Leucine
Serine	Tyrosine	
Methionine		
Proline		

The concentration of Class 1 amino acids is not very important (Pierce, 1987). During the first hours of fermentation, they produce a great number of carbon skeletons from amino acids in wort and during the final stages the keto acid analogues are synthesized from sugars. Thus, the initial concentration of these amino acids is not crucial since the products of this synthesis are being produced late in the fermentation period (Pierce, 1987). Proline has been grouped in class 1, as its concentration in wort is not important for yeast performance. In class 2, amino acid concentrations are essential, since during the later stages of fermentation, synthesis of the keto analogues from wort carbohydrates become the main sources of amino acid carbon skeletons (Pierce, 1987). Thus, although initially by-products are formed by sugars, they are further metabolized during the later stages of fermentation when by-products are no longer produced. Thus, changes in the concentration of Class 2 amino acids may affect the quality of the beer (Pierce, 1987). The carbon skeletons of Class 3 amino acids originate entirely from exogenous amino acids and there is very little contribution from sugar synthesis. Deficiency of these amino acids may induce major changes in the yeast nitrogen metabolism and also affect the final quality of the beer (Pierce, 1987).

1.6.1 Amino acids and other beer characteristics

The quality and stability of beer is related to the levels of certain amino acids and proteins present in wort (Gorinstein *et al.* 1999). Moreover, beer character and its organoleptic characteristics depend on the interaction between proteins, amino acids and polyphenols (Gorinstein *et al.* 1990 and Yokoi & Tsugita, 1988). The amount of proteins

and amino acids in beer are related to the technological processes of its preparation, especially during fermentation (Gorinstein *et al.* 1999).

High levels of amino acid residues in final beer may become nutrient sources for growth of microbial contaminants such as *Lactobacilli* and *Pediococci* (Gorinstein *et al.* 1999). The enantiomeric amino acid ratio of beers is a reliable parameter to assess quality, because it may indicate the quality of the technological process, adulteration, aging and shelf-life (Varadi *et al.* 1999). Proline and lysine are the most important amino acids in beer (Gorinstein *et al.* 1999). It is known that proline residues are responsible for the affinity towards proanthocyanidins and take part in the production of aromatic compounds that influence beer quality (Outtrup, 1989).

In addition, during kilning malt or during wort boiling, amino acids react with reducing sugars to form a wide range of reaction products, which add reducing properties together with colour and flavour to the beer (Lie, 1973). The individual amino acids differ in their effect on yeast metabolism and they also yield different reaction products when heated with reducing sugars (Lie, 1973).

1.6.2 The general amino acid permease (GAP) system

The regulation of amino acid uptake by brewer's yeast is complex involving carriers specific to certain amino acids (Jauniaux and Grenson, 1990). Over 20 specific transport systems mediate active transport of amino acids across the plasma membrane in *Saccharomyces cerevisiae* (Jauniaux and Grenson, 1990). Each is specific for just one or a few related L-amino acids (Jauniaux and Grenson, 1990). In addition to all these specific systems, *Saccharomyces cerevisiae* possesses a general amino acid transport system known as GAP (Surdin *et al*, 1965). The GAP system catalyses active transport of apparently all biological amino acids across the yeast plasma membrane and thus it is characterized as an enzyme amino acid permease (Palmqvist and Ayrapaa, 1969). It has the ability to concentrate exogenous amino acids up to 1000-fold. GAP transports wort amino acids by an active mechanism that involves proton co-transport (Palmqvist and Ayrapaa, 1969). The internal pool of a particular amino acid inhibits its own transport system, a phenomenon known as transinhibition (Walker, 1998). The amino acids compete with one another for their entry, a process that follows Michaelis kinetics. Different yeast strains have different Michaelis constants (Palmqvist and Ayrapaa, 1969). *GAP1* is believed to be the structural gene of the transporter. The encoded product of the

GAP1 gene is a highly hydrophobic polypeptide, as expected for an integral membrane protein, constituted by 601 amino acids (Jauniaux and Grenson, 1990). Forty one percent of these amino acids are hydrophobic and the N-terminal of the polypeptide is hydrophilic (Jauniaux and Grenson, 1990). Acidic and basic amino acids are less frequent in the sequence (Jauniaux and Grenson, 1990). Mutations at the *GAP1* locus affect the general amino acid permease system, by either modifying or repressing its affinity for specific substrates (Jauniaux and Grenson, 1990).

The GAP permease belongs to the class of single transport proteins, which mediate both substrate recognition and substrate translocation. It appears that the amino acid permease system first absorbs easily synthesized amino acids such as glutamine, asparagine and serine, whereas many with a long pathway of synthesis, especially the aromatic species, are taken up during the late stages of fermentation (Palmqvist and Ayrapaa, 1969). The affinity of the amino acid uptake system is higher for basic amino acids, followed by neutral and hydrophobic amino acids (Woodward and Cirillo, 1977). High levels of the most rapidly absorbed amino acids are favourable for the fast onset of fermentation. Based on various experimental investigations, it has been found that the uptake of amino acids from groups A (e.g. glutamic acid, aspartic acid, lysine, arginine) and B (e.g valine, methionine, leucine) are preferred by the yeast, with the result that the amino acids belonging in groups C (e.g. phenylalanine, tyrosine, tryptophan, alanine) and D (proline) can be detected in mature beer (Palmqvist and Ayrapaa, 1969).

The regulation of the general permease system is very complex and it has been found that its activity depends on the nature and composition of the nitrogen sources in the medium (Jauniaux and Grenson, 1990). In addition, the level of assimilable nitrogen present in wort profoundly affects nutrient uptake and subsequent fermentation performance (Walker, 1998). Also, the GAP transport system requires energy for operation and consequently the energy availability in the cell may limit the rate at which absorption can occur. In other words, the presence of an exogenous supply of certain nitrogenous nutrients inhibits the utilization of others by repressing the enzymes responsible for their assimilation.

Nitrogen limitation in yeast is known to result in a number of adaptations, which allow the cells to use alternate nitrogen sources (Woodward and Cirillo, 1977). Maximum activity of the GAP system is only expressed when nitrogen is limiting. In other words, GAP becomes effective in transporting D and L isomers of basic and neutral amino acids by the yeast only under nitrogen limiting conditions and also during cell growth in poor

nitrogen sources, such as proline (Woodward and Cirillo, 1977). During cell growth on proline, this amino acid is taken up by two permeases located in the plasma membrane, the GAP and the Put4p, the proline specific permease (ter Schure *et al.* 2000). Once these two proline transport proteins are properly expressed, they are activated by phosphorylation (ter Schure *et al.* 2000). Npr1p, a protein kinase homolog, is also involved in the activation of both proline permeases; this enzyme is responsible for phosphorylation of the protein transporters and hence their regulation and activation (ter Schure *et al.* 2000). Finally, when proline enters the cell it is then transferred from the cytoplasm into the mitochondria for its degradation (ter Schure *et al.* 2000).

The reactivation of the general amino acid uptake system during cell growth in poor nitrogen sources, such as proline or under nitrogen starvation in the presence of glucose and or fructose, results in an increase in the utilization rates of both hydrophobic and basic amino acids with simultaneous release of deaminated derivatives of hydrophobic amino acids, when the starved cells are inoculated in a nitrogen rich medium (Woodward and Cirillo, 1977). The deamination products released from the hydrophobic amino acids are presumed to be the α–keto acid and fusel oil derivatives, respectively, of the specific amino acids (Woodward and Cirillo, 1977). The manner in which nitrogen-starved yeast used hydrophobic amino acids in the presence of glucose and fructose illustrates how the general amino acid permease together with specific transaminases accomplish a selective retention of amino moiety of hydrophobic amino acids (Woodward and Cirillo, 1977). High transaminase activity is observed with tyrosine, phenylalanine, leucine and isoleucine as substrates, whereas aspartate, valine and methionine are only slowly transaminated (Woodward and Cirillo, 1977). Glycine is not transaminated at all. Transinhibition may account for the variation in amino acid transport activity during cell growth in repressed and derepressed cultures (Woodward and Cirillo, 1977). In addition, during nitrogen starvation conditions in the fermentation broth, an alternative control mechanism is also activated known as peptide transport system, which is described in more detail in a subsequent section (***1.7.2***).

On the other hand, when yeast cells are growing in wort containing an adequate utilizable nitrogen source, GAP system synthesis is inhibited and yeast appears to have a very high selectivity in terms of the amino acids that it utilizes as major nitrogen sources. For instance, in the presence of ammonia, glutamine and or asparagine excess, the

development of permease activity is prevented or decreases (Grenson and Acheroy, 1982; Grenson, 1983).

Two distinct control mechanisms are responsible for permease activity inhibition and these are the repression of permease synthesis and the reversible permease inactivation (Jauniaux and Grenson, 1990). Repression of GAP peptide synthesis involves the presence of ammonia in the medium, which regulates the *GAP1* messenger formation and stability (Jauniaux and Grenson, 1990). Hence, control of amino acid permease synthesis appears to result from transcript level repression (Jauniaux and Grenson, 1990). The *gdhCR* gene product is a repressor molecule and it controls the transcription of permease and enzyme genes, which are subjected of nitrogen catabolite repression. Glutamine is an effector of this regulation (Wiame *et al.* 1985). Mutations induced on this locus relieve the ammonia repression (Grenson *et al.* 1974).

When the nitrogen nutrients are good sources to support efficient yeast proliferation and viability, then the GAP transport system is dephosphorylated and subsequently deactivated and the Npr1p protein is responsible for triggering ubiquitin degradation, whereas the transporter membrane proteins via endocytosis are finally degraded inside the vacuole (ter Schure *et al.* 2000). In addition, the synthesis of new permeases is blocked at the level of gene expression (ter Schure *et al.* 2000). Hence, by supplying the fermentation medium with ample amounts of assimilable nitrogen, this leads to the gradual deactivation of the general amino acid transport system with continuous activation of individual amino acid transporters.

1.6.3 Amino acid biosynthesis

Yeast cells are capable of synthesizing all the necessary amino acids needed for growth from suitable carbon and inorganic nitrogen sources (**Figure 3**). The biosynthesis of amino acids is a complex process involving both carbon skeletons and nitrogen. The origins of these components vary according to the cultural conditions employed, each following an independent pathway (Jones *et al*. 1969).

Regulation of amino acid biosynthesis takes place on two levels, the regulation of enzyme formation by control of gene expression and the regulation of enzymatic activity that controls metabolic flux. Regulation of metabolic flux into selected pathways is affected by the enzymatic activity of coenzyme A (CoA) and glucose repression of

certain enzymes of amino acid biosynthesis, each of which provides linkage to carbon metabolism (Jones and Fink, 1982).

It is appears likely that an important aspect of the control of the metabolic flow is the exchange and interaction of metabolites between cellular compartments (Jones and Fink, 1982). Substantial portions of intracellular amino acids are compartmentalized within the cell, largely in the vacuole (Wiemken, 1980). Compartmentalization of amino acids within the mitochondria has also been found for some anabolic enzymes concerning mainly arginine and some branched-chain amino acid pathways (Jones and Fink, 1982).

With exception of the acidic amino acids, more than half the level of each amino acid is found in the vacuole. Moreover, the amino acid distribution within the cell varies with the wort amino acid content (Messenguy *et al*. 1980). For instance, if wort is supplemented with lysine, then amino acids are transferred from the vacuole into the cytosol. The opposite phenomenon occurs when ammonia concentration in the medium decreases (Jones and Fink, 1982).

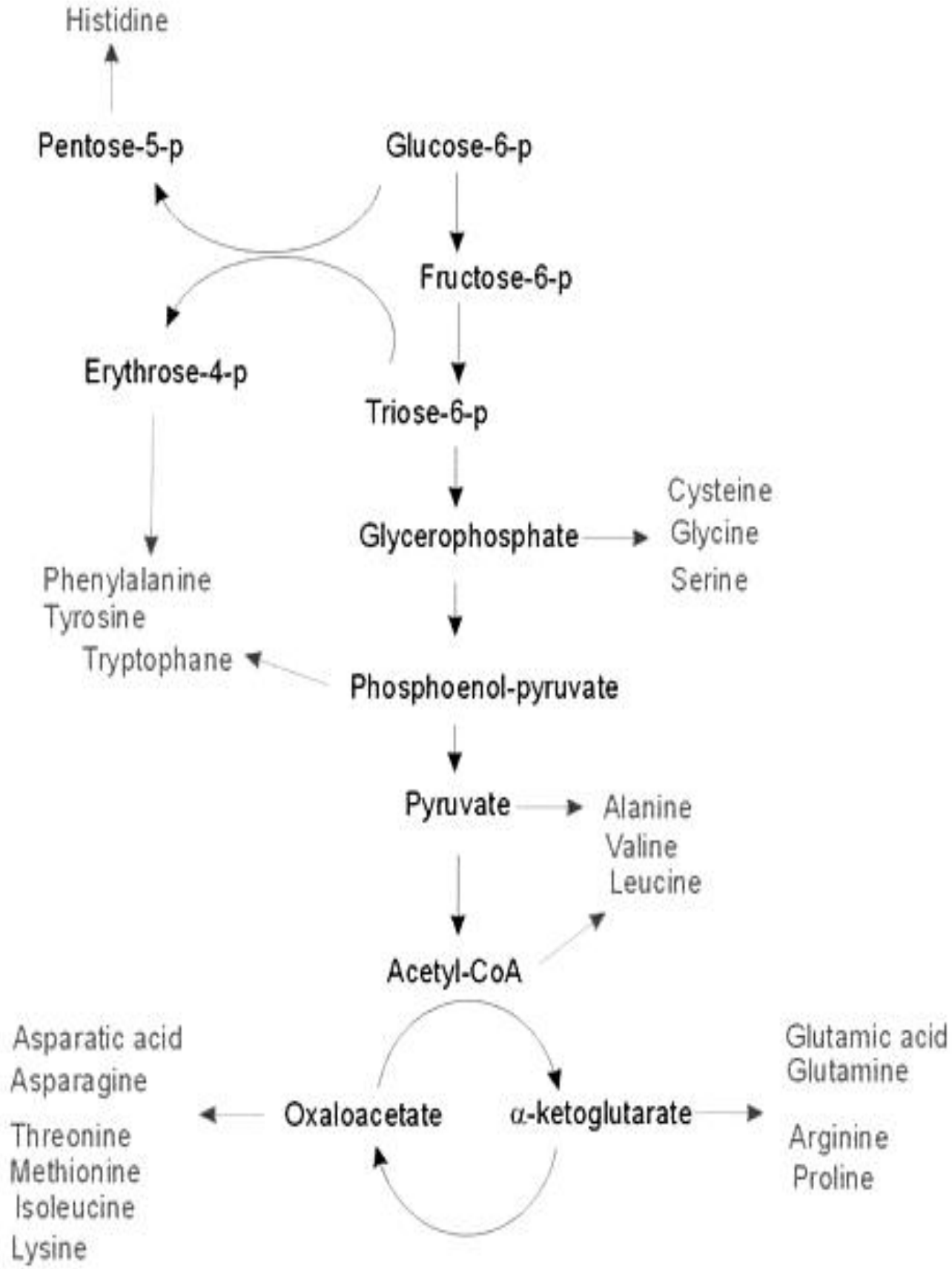


Figure 3: Amino acid biosynthetic pathways (Source: www.genome.ad.jp)

1.6.3.1 Glutamate family

The glutamate family includes amino acids that derive all or most of their carbon skeleton from glutamate or its immediate precursor, α–ketoglutarate: glutamate, glutamine, proline, arginine and lysine (Jones and Fink, 1982).

The biosynthesis of glutamate and glutamine (**Figure 4**) involves the following steps: glutamate dehydrogenase (GDH) catalyses an ammonia-dependent amination of α–ketoglutarate to produce glutamate (Jones and Fink, 1982). Two forms of the GDH are present in yeast the NADP and the NAD (Jones and Fink, 1982). The NADP dependent GDH is responsible for ammonia assimilation (Jones and Fink, 1982). Both the GDHs exist in the cytosol. Glutamate synthase, which is the enzyme that catalyses the NADPH linked reaction of α–ketoglutarate and glutamine to yield two molecules of glutamate, is present in very low levels in the yeast (Jones and Fink, 1982). The amino group of glutamate is then transferred to other amino acids by a mechanism called transamination (Jones and Fink, 1982).

Glutamine is synthesized from glutamate and ammonia in a reaction that requires energy in the form of ATP, catalysed by the enzyme glutamine synthetase (Jones and Fink, 1982). The NADP-GDH levels appear constitutive, although their concentration may be higher when ammonia rather than glutamate is the nitrogen source (Roon *et al*. 1974). Glutamate synthase levels are somewhat higher when yeast is growing in a medium with ammonia rather than glutamate (Roon *et al*. 1974).

Glutamine synthetase is essential in both anabolism and catabolism and its intracellular concentration depends on the quality and quantity of FAN (Jones and Fink, 1982). Glutamine synthetase levels are very low when glutamine is the sole yeast medium nitrogen source, intermediate when ammonia is the sole nitrogen source and high when poor nitrogen sources such as glutamate and proline are provided (Dubois and Grenson, 1974). The levels can be changed by varying the nutritional conditions (Jones and Fink, 1982).

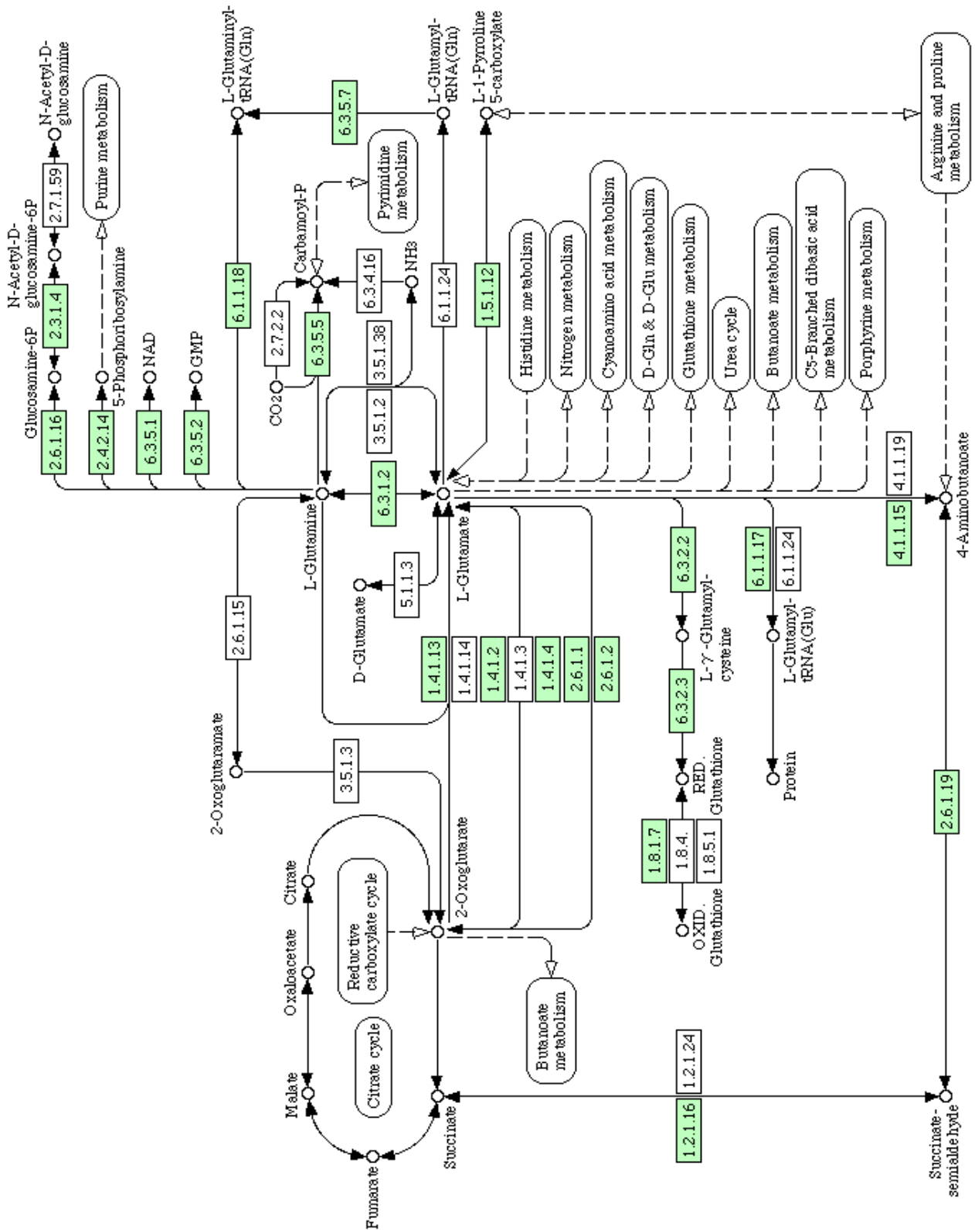


Figure 4: Glutamate and glutamine biosynthesis (Source: www.genome.ad.jp)

1.6.3.2 Proline biosynthesis

Proline synthesis (**Figure 5**) takes place by activation and reduction of the γ–carboxyl group of glutamate to yield glutamyl-γ-semialdehyde, which then spontaneously cyclizes to form pyrroline-5-carboxylate (P5C) (Jones and Fink, 1982). The P5C is finally reduced with the action of P5C reductase to give proline (Brandriss, 1979). This enzyme is also needed for arginine degradation (Brandriss and Magasanik, 1980). P5C reductase is present in the cytosol, whereas P5C dehydrogenase that carries out the catalysis of the oxidation of P5C to glutamate, is located in the mitochondria (Brandriss and Magasanik, 1981). The two different P5C pools apparently do not interfere with each other (Brandriss and Magasanik, 1980).

1.6.3.3 Arginine biosynthesis

The pathway from glutamate to arginine (**Figure 5**) involves activation and reduction of the γ–carboxyl group of glutamate (Jones and Fink, 1982). Before activation, in order for cyclisation of the glutamyl-γ-semialdehyde to be prevented, the α–amino group of glutamate is blocked with an acetyl group from acetyl-CoA. After transamination onto the aldehyde group of acetyl-γ-glutamyl semialdehyde to yield acetyl-ornithine, the acetyl group is transferred to glutamate and simultaneously ornithine and acetylglutamate are produced in order for the cycle to commence again. All the enzymes of this cycle are located in the mitochondria (Wipf and Leisinger, 1977).

After the completion of ornithine synthesis, the amino acid is transported outside the mitochondrion in order to react with carbomoylphosphate, a derivative of ATP, carbon dioxide and the amide group of glutamine, and citrulline is produced. The enzyme that acts as a catalyst in this conversion step of ornithine, is ornithine carbamoyltransferase (Jones and Fink, 1982). Then, aspartate reacts with the product of the above mentioned reaction and argininosuccinate is formed, followed by its break down into fumarate and arginine (Jones and Fink, 1982).

Acetylglutamate synthase is an enzyme that serves an anaplerotic function; it replenishes the supply of acetylglutamate as it decreases during the cell division (Jones and Fink, 1982). Acetylglutamate synthase is subject to end-product inhibition by arginine. This inhibition is synergistically reinforced by acetylglutamate, which can be considered as an

end product of the pathway (Jones and Fink, 1982). The enzyme is inhibited by CoA and this inhibition in addition with the inhibitory effect induced by arginine is coupled with carbon biosynthesis to carbon metabolism (Jones and Fink, 1982).

Acetylglutamate kinase is also an end-product that is inhibited by arginine synthesis. The levels of acetylglutamate kinase are rate-limiting for arginine production *in vivo* (Higler *et al*. 1973). Arginine, in order to induce feed back inhibition to the two mentioned enzymes must flow back into the mitochondria, where these two enzymes exist (Jones and Fink, 1982).

Two forms of carbamoylphosphate synthetase are present in the cytosol of yeast cells. The first form is repressed by growth in aginine-containing media, the other by growth in uracil-media. The level of the arginine specific carbamoylphosphate synthetase has been shown to be rate limiting for the arginine production *in vivo* when the pyrimidine specific enzyme is absent (Jones and Fink, 1982).

In the presence of moderate concentrations of arginine, the enzymes of arginine degradation including arginase are induced. Under these conditions, flow from glutamate in the pathway ceases. Arginase binds stoichiometrically to molecules of ornithine carbomoyltransferase in the presence of arginine and ornithine or citrulline (Messenguy *et al*. 1971). The complex dominates arginase catalytic activity but lacks catalytic activity for ornithine carbamoyltransferase. The anabolic pathway is interrupted, whereas the catabolic pathway remains intact (Jones and Fink, 1982).

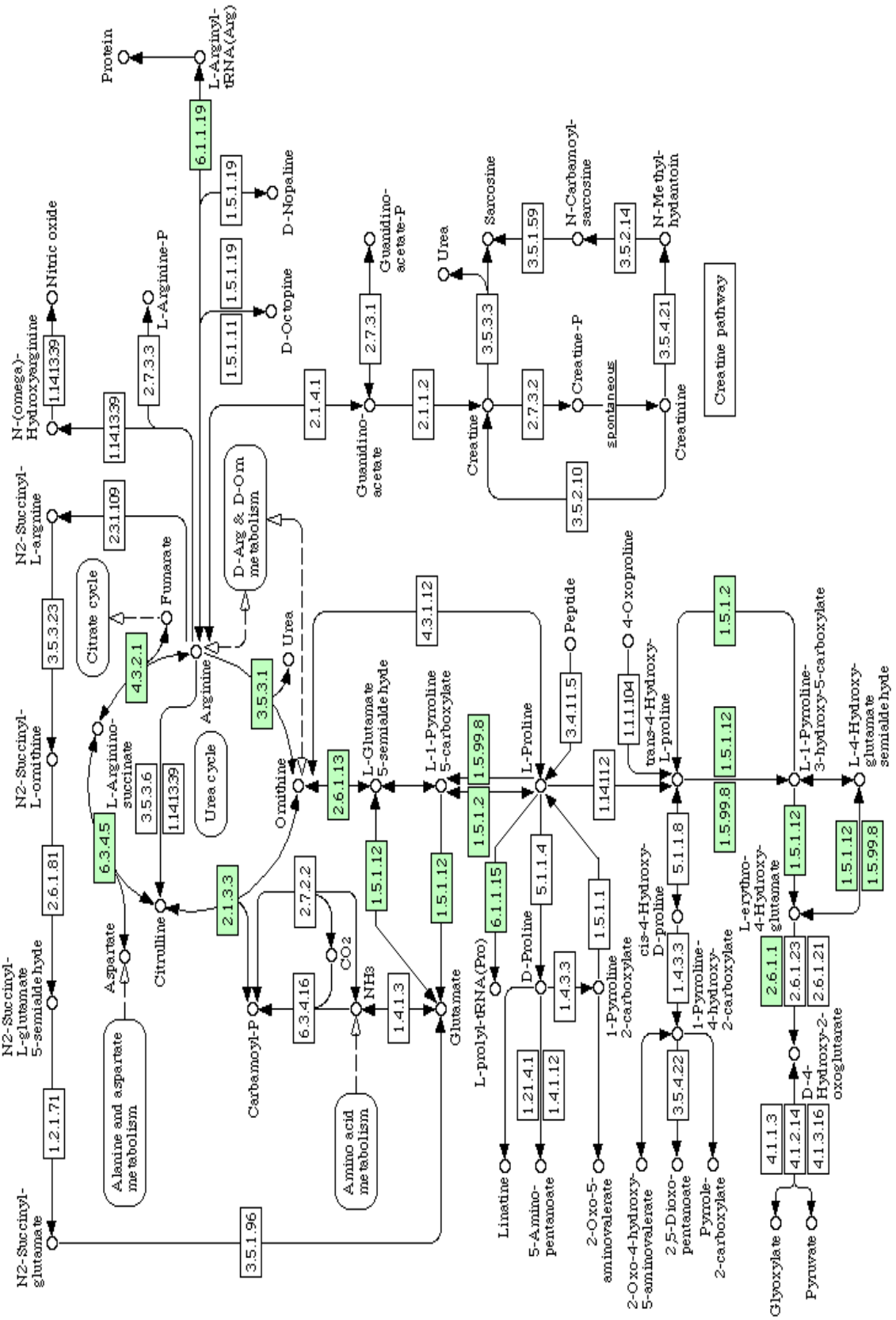


Figure 5: Proline and arginine biosynthesis (Source: www.genome.ad.jp)

1.6.3.4 Lysine biosynthesis

Yeast synthesizes lysine via the homocitric acid α–aminoadipic acid pathway (**Figure 6**). α–Ketoglutarate is condensed with acetyl-CoA to yield homocitrate, a reaction catalyzed by homocitrate synthase, which is present in two versions (Hinnebusch, 1992). Then, homocitrate is converted to α–ketoadipate, which is transaminated to yield α–aminoadipate followed by its conversion to α–aminoadipyl semialdehyde, in a three-step reaction leading to the formation of semialdehyde (Hinnebusch, 1992). Condensation of glutamate with semialdehyde and simultaneous reduction yields saccharopine, catalysed by saccharopine reductase (Hinnebusch, 1992). Saccharopine is eventually oxidized to yield lysine with concurrent release of α–ketoglutarate (Hinnebusch, 1992). The enzymes for the first steps of the metabolic pathway from homocitrate synthase to α–aminoadipate aminotransferase, are located in the mitochondria (Betterton *et al*. 1968).

Both of the types of homocitrate synthase enzymes are feedback-inhibited by lysine (Hinnebusch, 1992). Homocitrate synthase is reversibly inactivated by CoA (Hinnebusch, 1992). Protection against inactivation is afforded by α–ketoglutarate and lysine (Hinnebusch, 1992). Another step between the α–aminoadipate and lysine is also known to be feedback inhibited by the end product of the pathway (Hinnebusch, 1992).

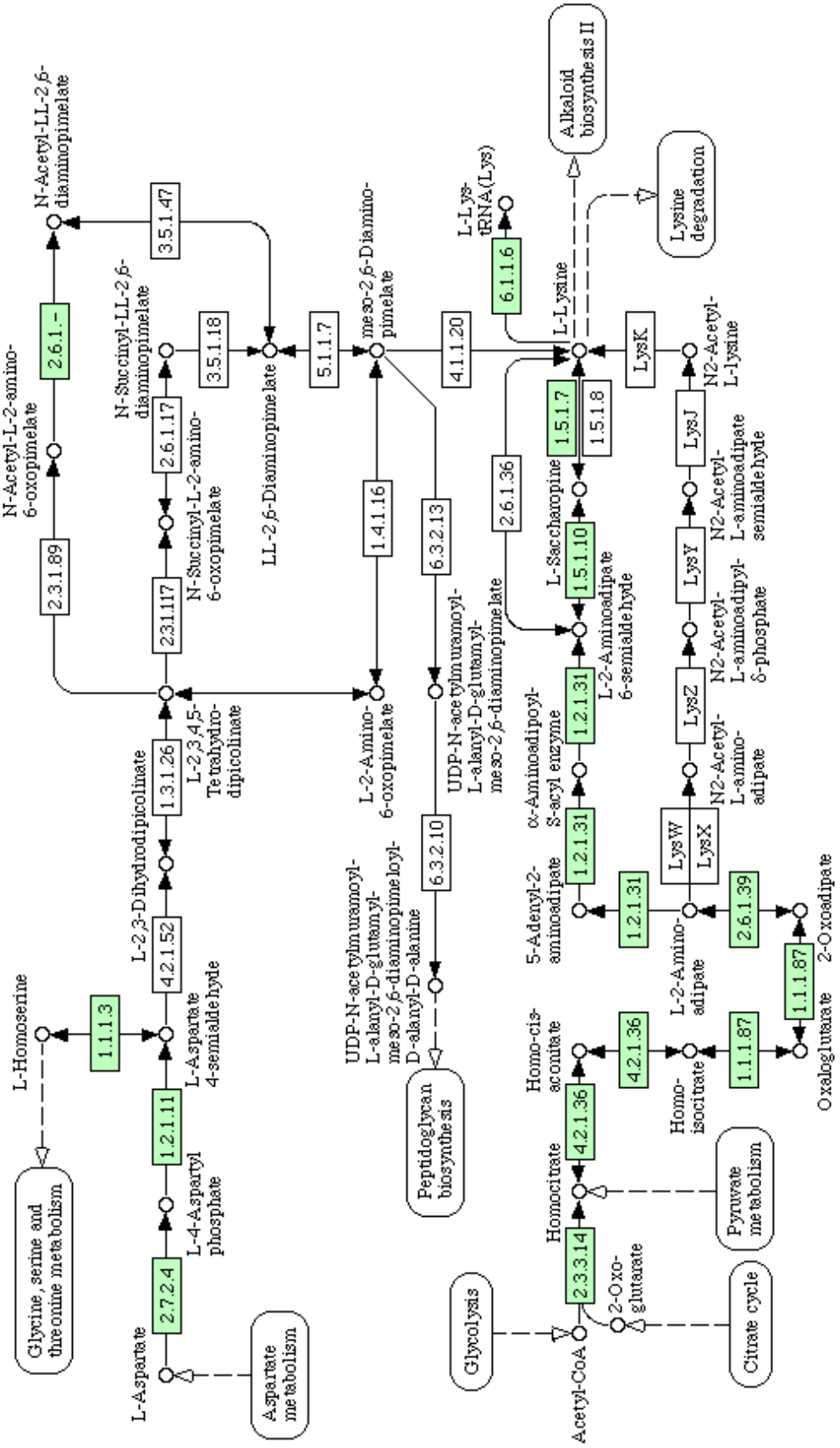


Figure 6: Lysine biosynthesis (Source: www.genome.ad.jp)

1.6.3.5 Aromatic amino acid biosynthesis (phenylalanine, tryptophan, tyrosine)

Synthesis of the aromatic amino acids proceeds via a common pathway to chorismate, at which point the pathway branches; one branch leads to tryptophan and the other to phenylalanine and tyrosine (**Figure 7**) (Hinnebusch, 1992). The common pathway to chorismate begins with condensation of erythrose-4-phosphate and phosphenolpyruvate to yield deoxy-D-arabino-D-heptulosonate-phosphate (DAHP) (Hinnebusch, 1992). In the next step, removal of the phosphate takes place and an internal redox reaction, which results in cyclization to yield 5-dehydroquinate (Hinnebusch, 1992). Dehydration, followed by reduction and then phosphorylation, yields shikimate phosphate, which is then condensed with a second molecule of phosphoenolpyruvate (Hinnebusch, 1992). Eventually, chorismate is formed by the removal of a phosphate with introduction of a second double bond (Hinnebusch, 1992). The branch reaction to phenylalanine and tyrosine progresses by rearrangement of chorismate to yield prephenate (Hinnebusch, 1992). Dehydration and decarboxylation of this metabolite leads to the formation of phenylpyruvate, the α–keto acid precursor of phenylalanine (Hinnebusch, 1992). Finally, transamination yields phenylalanine (Hinnebusch, 1992). Similarly, simultaneous dehydrogenation and decarboxylation of prephenate yields *p*-hydroxyphenylpyruvate, the α–keto acid precursor of tyrosine and again transamination leads to tyrosine synthesis (Hinnebusch, 1992). The other branch of the pathway, which results in tryptophan, commences with the conversion of chorismate to anthranilate (Hinnebusch, 1992). A phosphoribosyl group is transferred from phosphoribosylpyrophosphate onto the amino group of anthranilate (Hinnebusch, 1992). Isomerisation followed by cyclization yields indoleglycerolphosphate, which is first cleaved and indole condenses with serine for the production of tryptophan (Hinnebusch, 1992). The enzyme catalyzing this two-step reaction is tryptophan synthase (Hinnebusch, 1992). However, the exact number of aminotransferase enzymes that are involved in the transaminations of both phenylalanine and tyrosine is unknown (Hinnebusch, 1992). In the absence of aromatic amino acids (phenylalanine, tyrosine, and tryptophan) or under low concentrations, chorismate follows the tryptophan pathway (Hinnebusch, 1992). This occurs because of the kinetics of the enzymes, antranilate synthase and chorismate mutase and their relative affinities for the common substrate, chrorismate (Hinnebusch, 1992). Anthranilate

synthase is feed-back inhibited by tryptophan and this inhibition is counteracted by chorismate (Hinnebusch, 1992).

Flow into the phenylalanine-tyrosine sub-pathway proceeds via chorismate mutase, when the levels of substrates are adequate (Hinnebusch, 1992). This enzyme is feed-back inhibited by tyrosine but surprisingly not by phenylalanine, whereas the tyrosine induced inhibition is deactivated by tryptophan (Hinnebusch, 1992). Thus, flow of chorismate into the tyrosine-phenylalanine formation path is enhanced when tryptophan is in excess, because it inhibits flow of chorismate into the tryptophan branch by inhibiting anthranilate synthase and activates chorismate mutase (Hinnebusch, 1992).

Prephenate is the common substrate leading to either phenylalanine or tyrosine (Hinnebusch, 1992). Two enzymes are responsible for the conversion of this metabolite to the final amino acids and based on their kinetics and affinities for prephenate, prephenate passes into either phenylalanine or tyrosine (Hinnebusch, 1992). Prephenate dehydratase is involved in phenylalanine biosynthesis, whilst prephenate dehydrogenase in tyrosine formation (Hinnebusch, 1992). Prephenate dehydratase is feedback inhibited by phenylalanine, whereas prephenate dehydrogenase by tyrosine (Hinnebusch, 1992). Thus, when phenylalanine is in excess, the flux of phephenate is diverted into tyrosine synthesis, because the activity of the dehydratase enzyme is inhibited (Hinnebusch, 1992). When phenylalanine is present at low levels, prephenate is converted into phenylalanine, whether or not tyrosine is present, because of the low affinity of the dehydrogenase enzyme for prephenate (Hinnebusch, 1992).

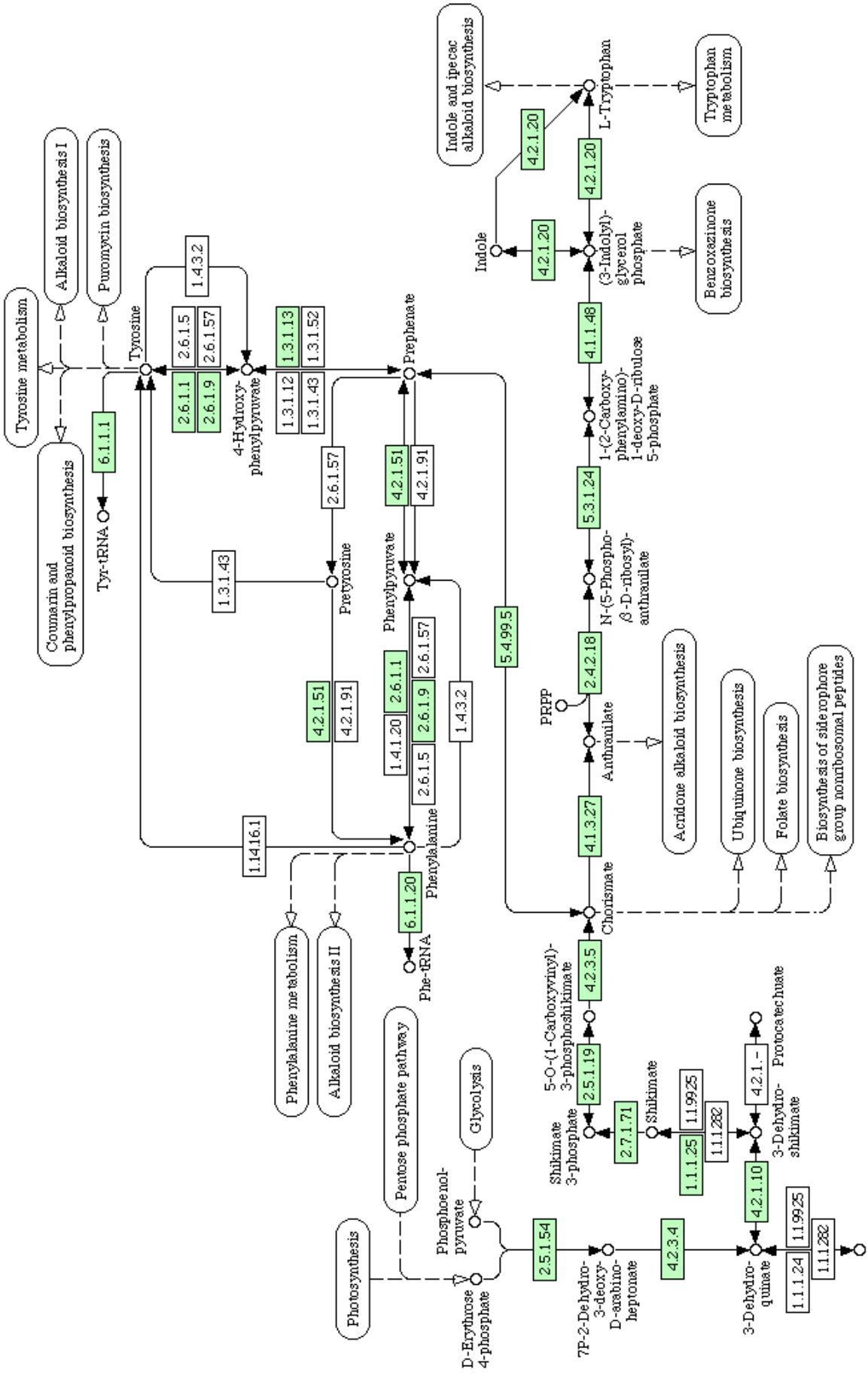


Figure 7: Phenylalanine, tyrosine and tryptophan biosynthesis (Source: www.genome.ad.jp)

1.6.3.6 Serine family biosynthesis (serine, glycine, cysteine)

The serine amino acid family consists of serine, glycine and cysteine (Jones and Fink, 1982). Serine biosynthesis proceeds via the phosphorylated pathway (**Figure 8**). To be more specific, the substrate 3-P-glycerate is converted into 3-P-hydroxypyruvate with the action of the enzyme phosphoglycerate dehydrogenase. Then, a molecule of glutamate transaminates its amino group to the 3-P-hydroxypyruvate, which is converted into O-phosphoserine. Finally, this metabolite is converted into serine by removal of the phosphate group (Jones and Fink, 1982).

Glycine can be generated from serine by serine hydroxymethyltransferase (**Figure 8**) (Jones and Fink, 1982). Two species of this enzyme exist; one can be found in the cytosol and the other in the mitochondria. The levels of this enzyme activity may rise in response to glycine supplementation (Jones and Fink, 1982).

For cysteine synthesis, two pathways are possible. The first proceeds by acetylation of serine, a reaction that yields *O*-acetylserine, followed by sulfhydrylation to yield cysteine (**Figure 9**) (Jones and Fink, 1982). Two enzymes are responsible for this transformation. One catalyses only cysteine synthesis (cysteine synthase) and the other catalyses both cysteine and homocysteine synthesis (homocysteine-cysteine synthase) (Jones and Fink, 1982).

The second pathway proceeds from homocysteine by condensation with serine to yield cystathionine (**Figure 11**). This reaction is catalysed by β−cystathionine synthase, followed by the cleavage catalysed by γ−cystathionase resulting in the formation of cysteine (**Figure 11**) (Jones and Fink, 1982). Alternatively, the substrate of β−cystathionine synthase may be homocysteine and *O*-acetylhomoserine (**Figure 11**), rather than homocysteine and serine. The homocysteine-cysteine is inhibited by methionine when *O*-acetylhomoserine is the substrate (**Figure11**) (Jones and Fink, 1982).

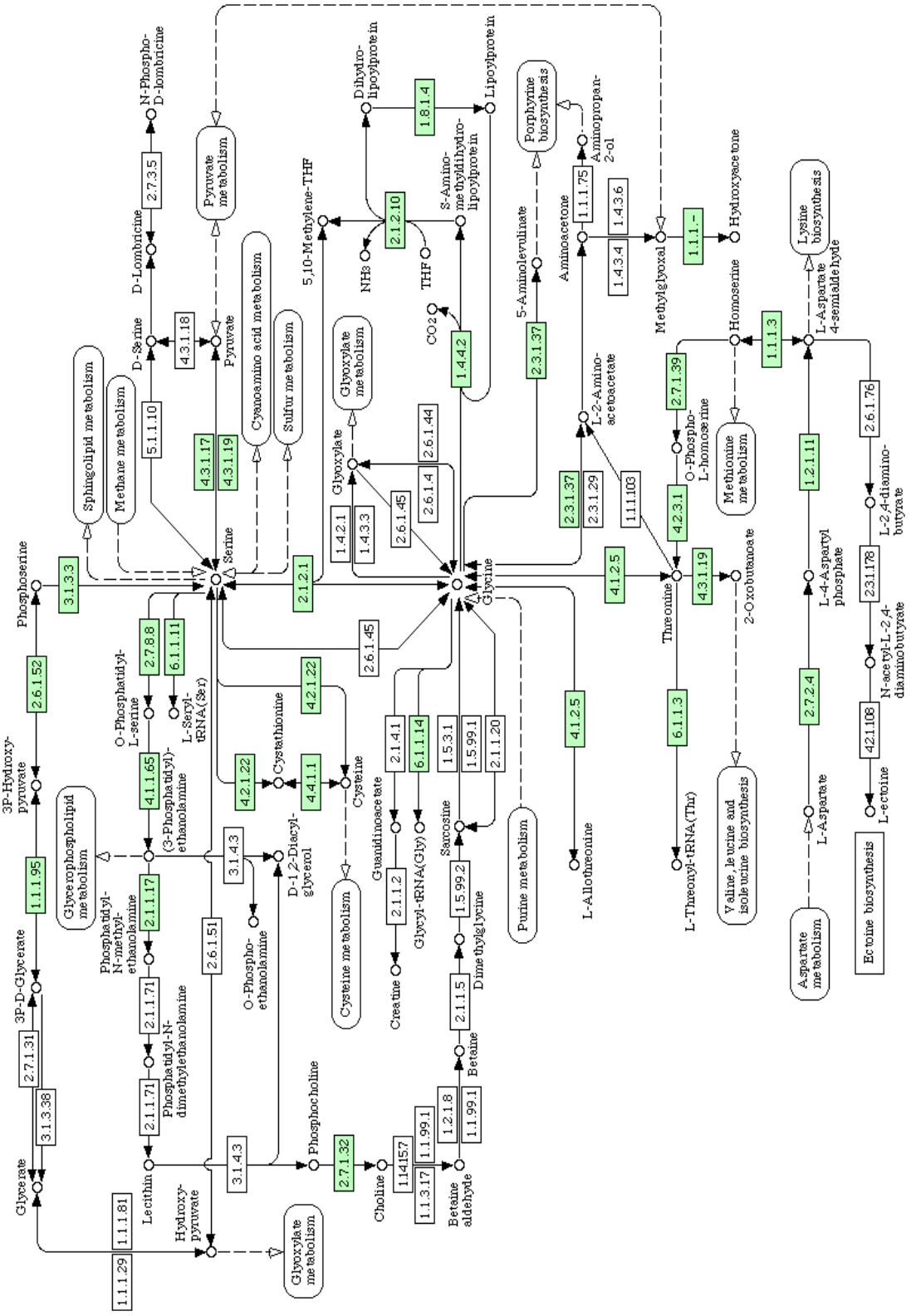


Figure 8: Glycine and serine biosynthesis (Source: www.genome.ad.jp)

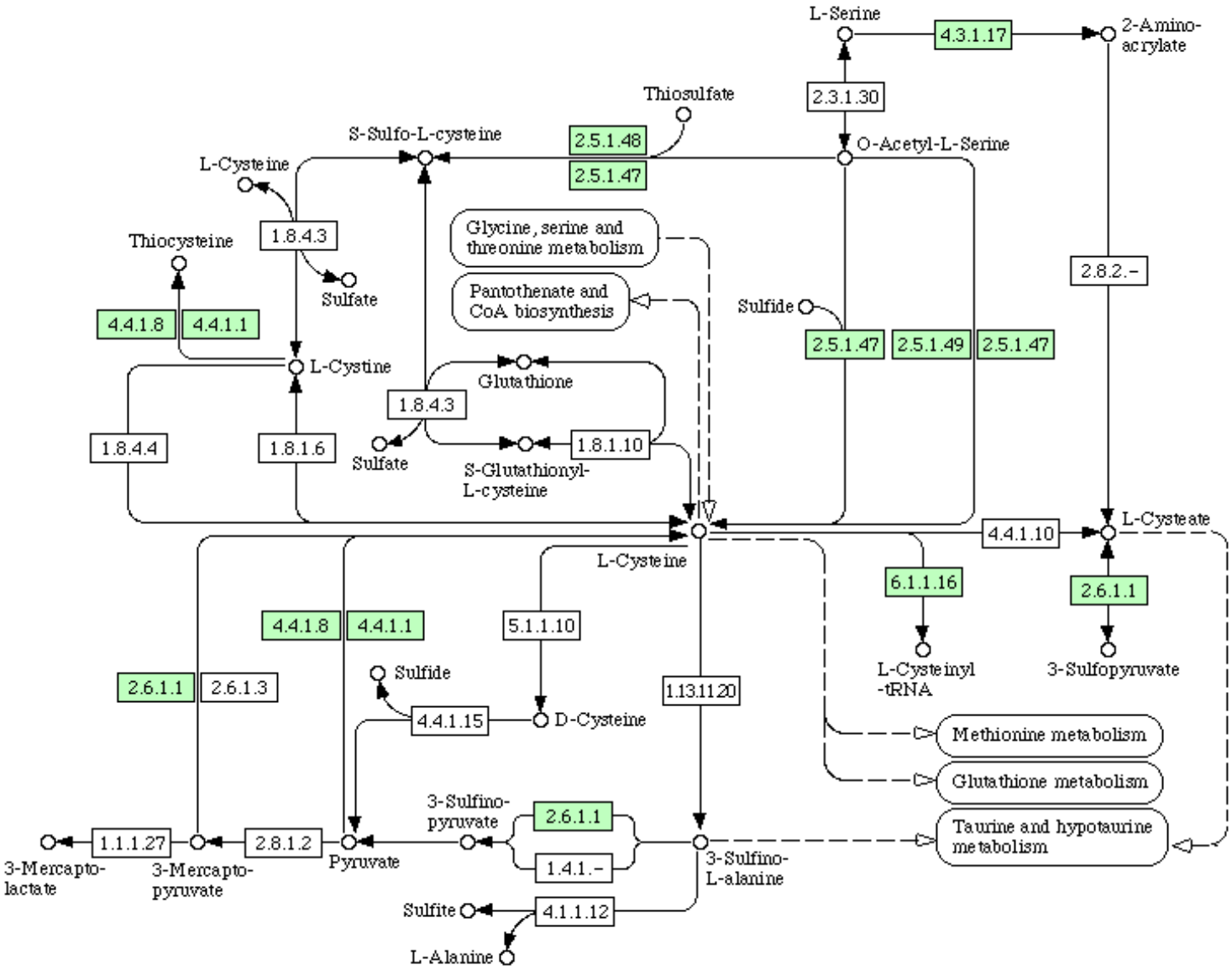


Figure 9: Cysteine biosynthesis (Source www.genome.ad.jp)

1.6.3.7 Aspartate family biosynthesis (aspartate, asparagine, threonine, methionine, isoleucine)

The aspartic acid family of amino acids includes aspartate, asparagine, threonine, methionine and isoleucine (Jones and Fink, 1982). Synthesis of aspartate occurs by transamination from glutamate into oxalacetate (**Figure 10**) (Jones and Fink, 1982). The enzyme catalyzing this reaction, aspartate aminotransferase is neither repressed not inhibited by methionine, threonine or aspartate.

Asparagine (**Figure 10**) derives from aspartate, in a glutamine-dependent reaction (Jones and Fink, 1982). Two isozymes of asparagine synthetase exist and both these enzymes are inhibited by asparagine and both are repressed when asparagine synthesis is limiting for growth (Jones and Fink, 1982).

A common pathway for synthesis of both threonine and methionine (and eventually isoleucine) consists of three reaction steps leading from aspartate to homoserine (Jones and Fink, 1982). The β–carboxyl of aspartate is activated by a reaction catalysed by aspartokinase, followed by reduction of β–aspartyl-phosphate to aspartate semialdehyde. This reaction step is catalysed by aspartate semiladehyde dehydrogenase. Reduction of the semialdehyde, catalyzed by homoserine dehydrogenase, yields homoserine. From this point the pathways for methionine and threonine biosynthesis diverge (Jones and Fink, 1982).

For threonine biosynthesis, the end product of the above mentioned pathway is phosporylated by homoserine kinase and *O*-phosphohomoserine is formed (Jones and Fink, 1982). Then, it is hydrolyzed in a reaction catalyzed by threonine synthase to finally yield threonine. Flow of the pathway into the threonine branch is controlled by end product inhibition of homoserine kinase by threonine. Levels of homoserine kinase are unaffected by threonine addition to the medium, but are induced by addition of methionine.

Biosynthesis of methionine (**Figure 11**) commences with an acetyl-CoA-dependent acetylation of homoserine to yield *O*-acetylhomoserine (Jones and Fink, 1982). Then two routes are possible for conversion of *O*-acetylhomoserine to homocysteine. The first is direct sulfhydrylation of the substrate to yield homocysteine, with the sulfhydryl donor, free sulfide. The second route involves condensation of *O*-acetylhomoserine with cysteine to produce cystathionine, a reaction catalyzed by γ–cystathionine synthase,

followed by its cleavage by β–cystathionase and the derivatives of this reaction are homocysteine and pyruvic acid. Homocysteine, finally acts as the acceptor for a methyl group donated by 5-tetrahydropteroyl-triglutamate and yields methionine (Jones and Fink, 1982).

The sulfhydryl donor, sulfide, is generated by reduction of sulfate. Sulfate reacts first with ATP in a two step reaction to form an intermediate product known as 3-P-adenylylsulfate (PAPS), which is then reduced to sulfite by the enzyme PAPS reductase. Sulfite then is reduced to sulfide by sulfite reductase and sulfide is donated to the acetylated amino acid acceptors (*O*-acetylhomoserine and *O*-acetylserine) to yield eventually homocysteine and cysteine, respectively. Homocysteine directly receives a methyl group to yield methionine, whilst cysteine is first coverted into cystathionine and then into homocysteine and then it is also methylated resulting in the derivation of methionine (**Figure 11**) (Jones and Fink, 1982).

Isoleucine biosynthesis (**Figure 12**) starts with deamination of threonine (threonine deaminase) to first yield α–ketobutyrate, which with pyruvate both serve as acceptors of an aldehyde derived by thiamine pyrophosphate to yield acetohydroxy acids, acetolactate and α–acetohydroxybutyrate, which are precursors of valine and isoleucine, respectively (Jones and Fink, 1982). These reactions are catalyzed by the enzyme acetohydroxy acid synthase (AHAS). The two acetohydroxy acids undergo isomerization and then reduction, by a single isomeroeductase and dihydroxy acid precursors for valine and isoleucine formation are derived (Jones and Fink, 1982). The α,β–dihydroxy acids are then dehydrogenated leading to α–ketoisovalerate and α–keto-β–methylvalerate, which are the α–keto acid precursors of valine and isoleucine, respectively (Jones and Fink, 1982). The enzyme dihydroxy acid dehydratase acts as the catalyst of this reaction. Finally, the keto acids are transaminated to yield valine and isoleucine (Jones and Fink, 1982). All the enzymes discussed required for synthesis of the α–keto precursors leading to valine and isoleucine formation originating from threonine and pyruvate, are located in the mitochondria. The amino donor for the final transamination step has not been identified.

Isoleucine activates the enzyme threonine deaminase, responsible for the deamination of this particular amino acid, when isoleucine levels are low in the wort and vice versa (Jones and Fink, 1982). The isoleucine inhibition induced by high amino acid levels in the wort is reversed by valine (Jones and Fink, 1982). Thus, equilibrium of these two amino acids in the wort is essential. In addition, the enzyme AHAS catalyzing the

reactions leading to the production of valine and isoleucine precursors, plays a significant role to which amino acid biosynthetic pathway will be followed (Jones and Fink, 1982). To be more specific, at low threonine wort concentrations, little α–ketobutyrate is synthesized due to the inactivation of the enzyme threonine deaminase (Jones and Fink, 1982). Hence, the AHAS enzyme is directed to the valine synthesis pathway, since ample pyruvate is available in the medium (Jones and Fink, 1982). On the other hand, high threonine levels in the wort result in the formation α–ketobutyrate with simultaneous activation of the enzyme deaminating threonine (Jones and Fink, 1982). Therefore, the pathway is lead in the direction of isoleucine formation, since the above mentioned substrate is the precursor for isoleucine synthesis.

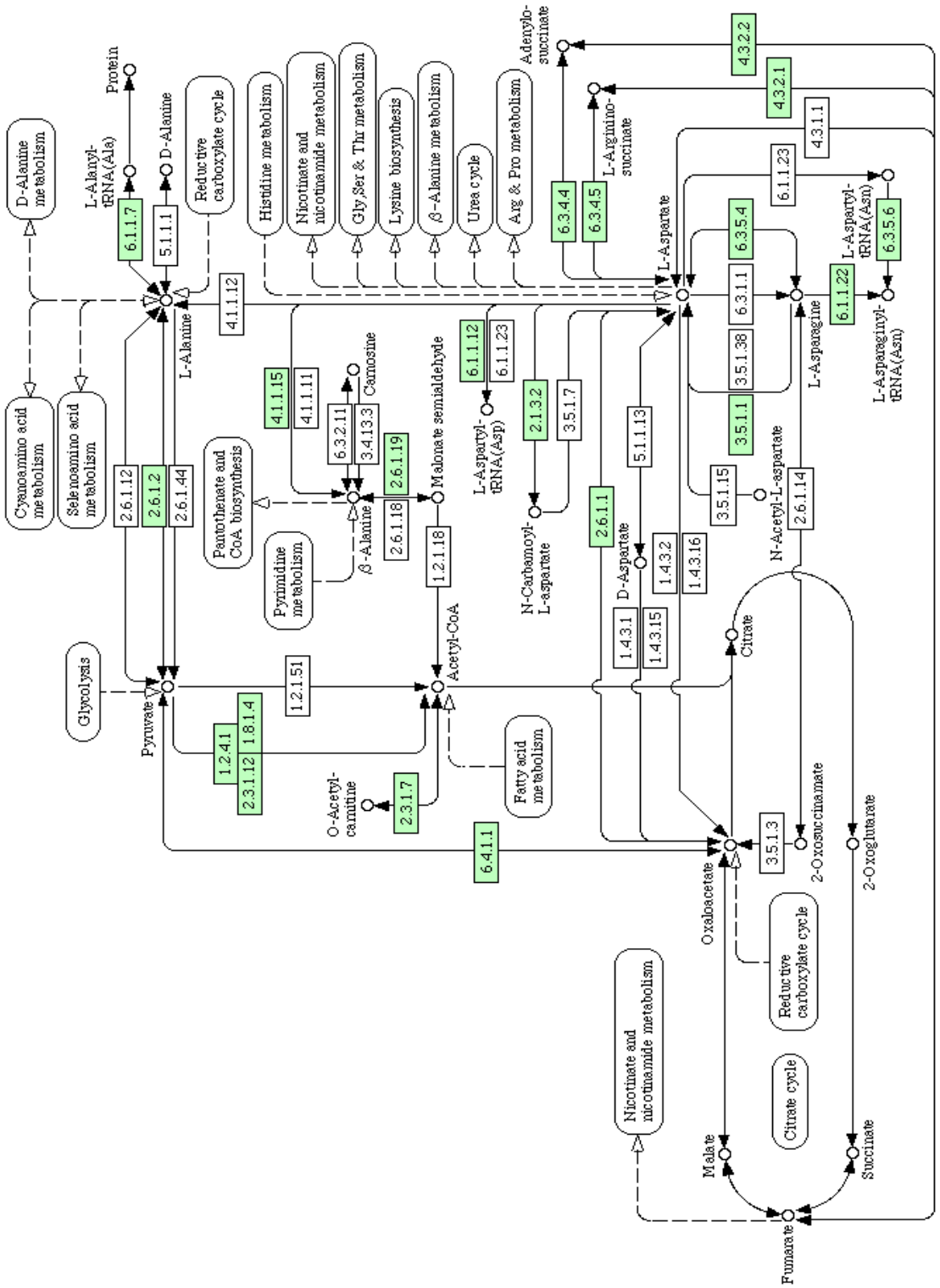


Figure 10: Asparagine, aspartate and alanine biosynthesis (Source: www.genome.ad.jp)

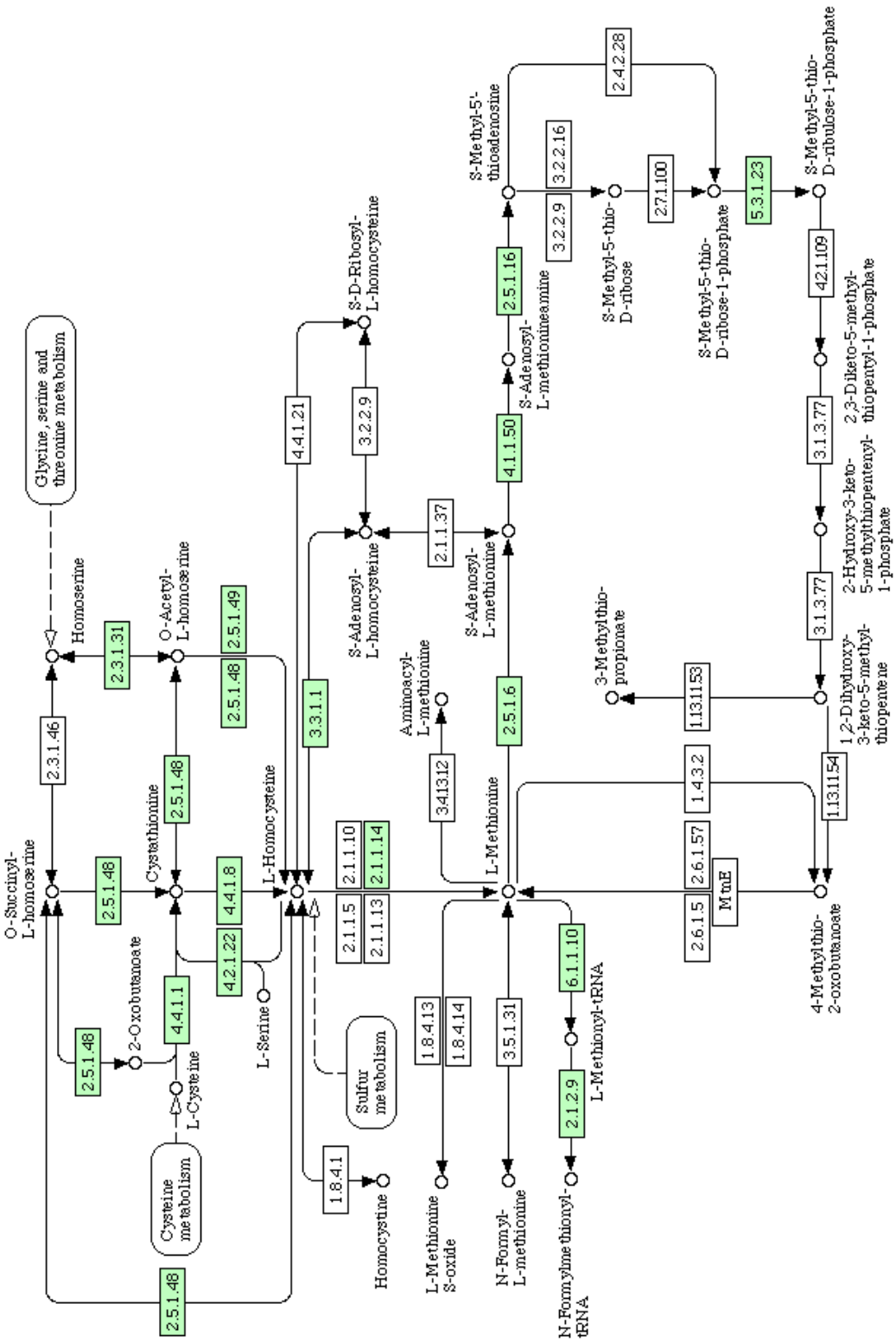


Figure 11: Methionine and cysteine biosynthesis (Source: www.genome.ad.jp)

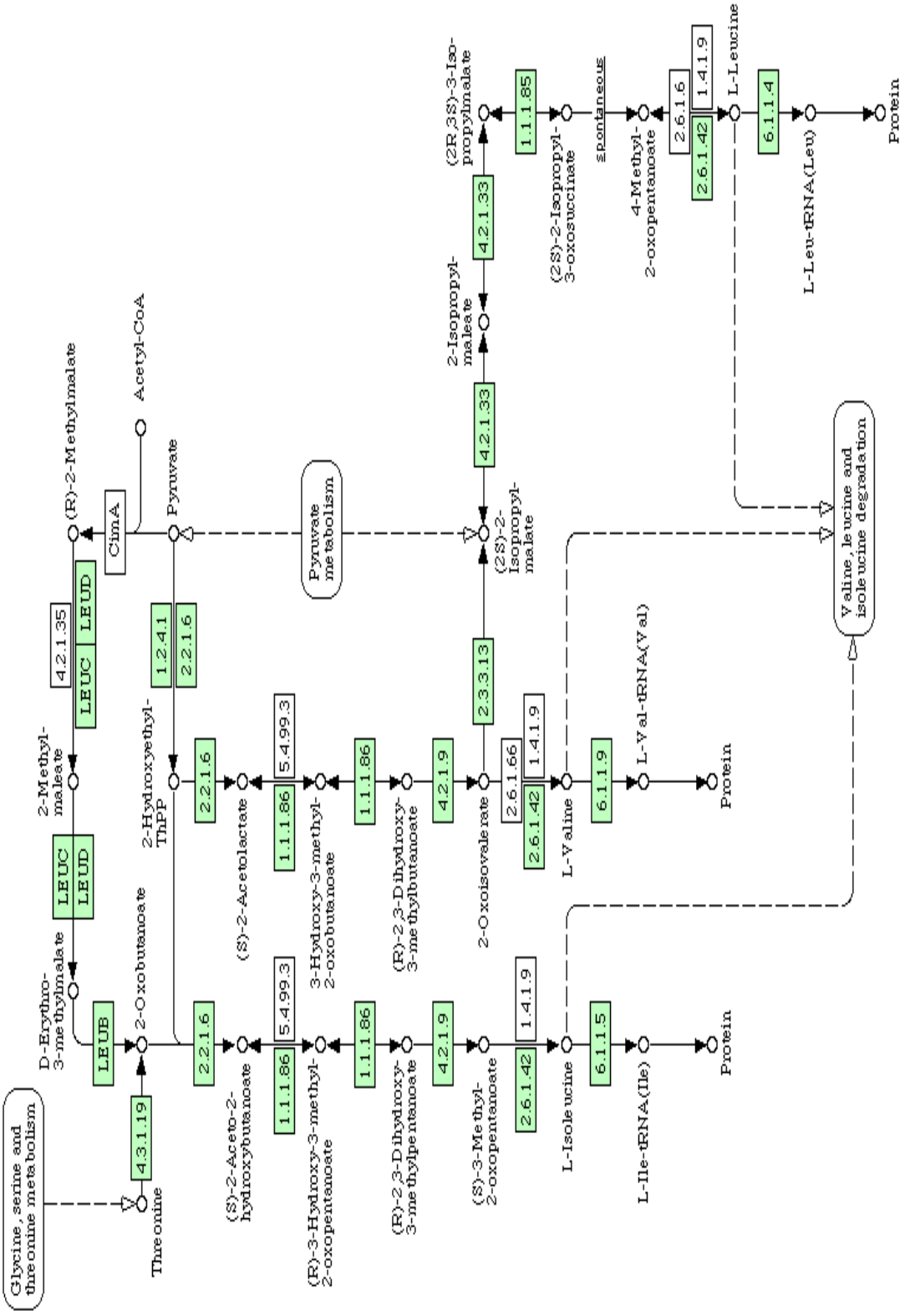


Figure 12: Valine, leucine and isoleucine biosynthesis (Source: www.genome.ad.jp)

1.6.3.8 Pyruvate family biosynthesis (valine, leucine, alanine)

The pyruvate family of amino acids includes alanine, valine and leucine which are so called because a major portion of their carbon skeletons derives from pyruvic acid (Jones and Fink, 1982). As it was described in the previous paragraph, valine biosynthesis (**Figure 12**) involves binding of pyruvate with an aldehyde thiamine pyrophosphate derivative to yield acetolactate, the precursor of valine (Jones and Fink, 1982).

The α–keto acid precursor of valine, α–ketoisovalerate, is the source of most of the carbon skeleton of leucine (**Figure 12**), because it is a substrate for a condensation reaction with acetyl-CoA, catalyzed by α–isopropylmalate (IPM) synthase. α–IPM undergoes isomerization to β–IPM, followed by oxidative decarboxylation to yield the α–keto acid precursor of leucine, α–ketoisocaproate (Jones and Fink, 1982). The final step in leucine biosynthesis involves the transamination of the α–keto acid as described in both valine and isoleucine formation (Jones and Fink, 1982). Flow of carbon into the leucine pathway appears to be controlled by feedback inhibition of α–IPM synthase by leucine (Jones and Fink, 1982).

Very little information about the biosynthesis of alanine is available (**Figure 10**), but it is presumed that its formation occurs by transamination of pyruvic acid.

1.6.3.9 Histidine biosynthesis

Biosynthesis of this particular amino acid (**Figure 13**) initiates with condensation of the ribosylphosphate moiety of phosphoribosyl-PP (PRPP) onto ring nitrogen 1 of ATP to yield phosphoribosyl-ATP (PR-ATP) (Jones and Fink, 1982). Pyrophosphorolysis of PR-ATP then gives phosphoribosyl-AMP (PR-AMP), which is followed by the opening of the purine ring between the N1 and C6, forming a compound referred as phosphoribosylformimino-5-aminoimidazolecarboxamide ribonucleotide (BBMII). The ribose moiety in BBMII that originated from PRPP undergoes isomerization to the ribulose group of BBMIII (Jones and Fink, 1982). Amino transfer from glutamine and closure of the new imidazole ring results in the formation of two new metabolites known as imidazoleglycerolphosphate and the purine intermediate 5-phosporibosyl-5-amino-4-imidazole-carboxamide (AICAR). Dehydration of imidazoleglycerolphosphate yields imidazoleacetolphosphate, which then undergoes transamination to histidinolphosphate.

Histidinol is then produced by dephosphorylation of the above metabolite, which is then oxidized in two steps to eventually form histidine. Flow of carbon into the pathway is regulated by feedback inhibition by histidine of the phosphoribosyltransferase, which is the transferase enzyme responsible for the first reaction of the pathway (Jones and Fink, 1982).

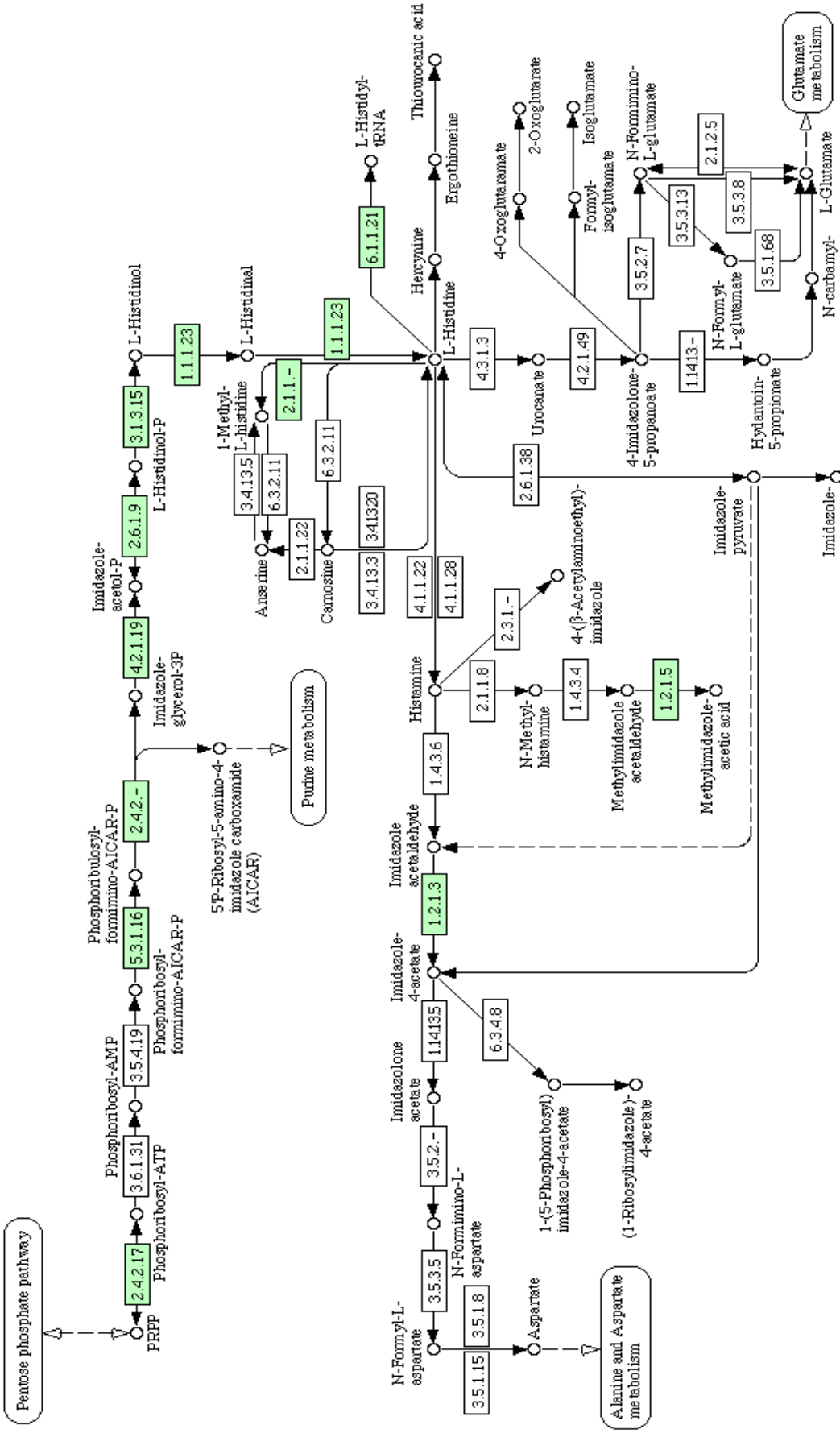


Figure 13: Histidine biosynthesis (Source: www.genome.ad.jp)

1.7 Oligopeptides

In contrast to amino acid utilization, investigations into the role of small peptides in yeast nitrogen requirements are still in their infancy, although the utilization of small peptides by brewing yeasts was originally confirmed prior to the 1950's (Dalme and Thorne, 1949). Approximately 30% of incorporated nitrogen compounds come from sources other than amino acids (Takahashi *et al.* 1997). Small peptides can be also used as nutritional sources of amino acids, as carbon or nitrogen sources and as precursors of cell wall peptides during yeast growth (Perry *et al.* 1994). Although, the uptake and metabolism of small peptides only affect higher alcohol production indirectly, their effects on beer organoleptic characteristics can not be ignored (Takahashi *et al.* 1997). Little is known about wort peptides and their uptake, even though enhancement of the ability of yeast to utilize small peptides may enable worts with low FAN content to be fermented without restricting yeast growth and sugar attenuation (Calderbank *et al.* 1985).

Four hundred dipeptides and up to 8,000 possible tripeptides may theoretically be found in wort, according to the number of amino acids in wort and their binding combinations to form oligopeptides with two or three residues (Macwilliam and Clapperton, 1969). Approximately, 40% of the oligopeptide fraction is removed by yeast during fermentation and the peptides remaining in beer differ from those found in wort (Calderbank *et al.* 1985).

Very little is known about the range of peptides found in wort or the order in which they are removed (Calderbank *et al.* 1985). Most brewing yeast strains transport peptides with no more than 3 residues (Calderbank *et al.* 1985). Peptide formation in malted barley usually increases by the third day of germination and decreases at the fourth, whereas it increases again on day five (Agu, 2003). Decrease of peptide release could be due the migration of nitrogen in the form of peptides from the storage endosperm to the embryo and to the roots and shoots (Agu, 2003).

1.7.1 Oligopeptide utilization

Ingledew (1975) reported that yeasts are capable of absorbing small peptides, although their growth is much slower when small peptides are the sole nitrogen sources. Polypeptides are also used, as yeasts elaborate levels of proteolytic enzymes

extracellularly, which ultimately act to provide additional assimilable nitrogen to the cells (Ingledew, 1975). Yokota *et al.* (1993) and Hickman and Buckee (1982) have also reported that the concentration of low molecular weight peptides was seen to be reduced during brewing fermentations. It has also been proven that the L-steroisomers, but not the D-forms of amino acids in di- and tripeptides are preferred substrates and also the basic amino acid-containing peptides are transported more rapidly than peptides that consist of acidic amino acids (Ingledew and Patterson, 1999).

A size limit of two to three amino acid residues exists in peptides transported in *Saccharomyces cerevisiae*, but this limit is strain dependent (Marder *et al*. 1977). Based on that, by studying fermentations that were carried out with a malt extract (a wort type prepared from malted cereals in a concentrated form), a very low attenuation rate was observed compared to that of the standard wort, even when the initial FAN levels were 200mg/L (Paik *et al*. 1991). This suggested that regardless of the high initial FAN content of the malt extract, peptides larger than tripeptides are not utilized by yeasts (O'Connor and Ingledew, 1989). The inability of a larger peptide to enter the yeast cells, suggests a size limit for peptide transport (Marder *et al.* 1977).

The uptake of small peptides from the growth medium is carrier mediated, energy dependent and of broad substrate specificity (Island *et al.* 1983). The initial rate of uptake of oligopeptides in brewing yeasts is higher and more efficient when the cells were propagated in a medium containing poor assimilable nitrogen sources, such as proline or when ammonium ions were in limiting amounts in the medium (Island *et al.* 1987; Nisbet and Payne, 1979; Ingledew and Patterson, 1999). These results have been confirmed by Nisbet and Payne (1979), suggesting that nitrogen repression appears to play a major role in the regulation of peptide transport in yeasts.

Patterson and Ingledew (1999) suggested that the phase of growth and the concentration of non-peptide nitrogen might also affect peptide utilization. Dipeptide transport in brewing yeasts is affected by the presence of micromolar concentrations of amino acids in the growth medium (Island *et al*. 1987). The presence of amino acids in the growth medium increases the sensitivity of yeast to small peptides, but not all amino acids produced the same response to the uptake of small peptides (Island *et al*. 1987). Leucine and tryptophan appear to be the most effective peptide uptake regulators regardless of their concentration (Island *et al.* 1987; Nisbet and Payne, 1979; Ingledew and Patterson, 1999). On the other hand, asparagine appears to be a potential inhibitor of peptide utilization (Ingledew and Patterson, 1999). For that reason, three categories of amino

acids have been identified based on the effect that they induce on the uptake of small peptides (Island *et al*. 1987). These are amino acids that promote only slight sensitivity, some amino acids that are good nitrogen sources (e.g. arginine) and they repress the yeast's sensitivity to small peptides and other amino acids that are considered as inducers or accelerators of yeast peptide uptake sensitivity (Island *et al*. 1987). Examination of the intracellular pools of cells grown in a medium containing amino acid inducers and small peptides showed increased levels of the peptide amino acid residues (Nisbet and Payne, 1979). This observation leads to the speculation that these accumulated residues might bring transinhibition of the amino acid uptake (Island *et al*. 1987). However, peptides and amino acids do not compete directly for uptake and this is because separate transport mechanisms are used (Nisbet and Payne, 1979).

In addition, Moneton *et al.* (1986) working with toxic and radiolabelled di- and tripeptides as medium supplements, suggested that the yeast affinity for exogenous peptide transport is highly dependent on the nature of the peptide. As it was observed from their investigation, di- and tripeptides that constitute methionine are actively transported into the cells, whereas equal numbers of residual peptides, containing glycine were not preferred by the yeast (Moneton *et al.* 1986). Marder *et al.* (1977) also corroborated these observations by saying that the growth responses observed for peptides of different sequence might reflect variations in affinity for the peptide transport system. In addition, the amino acid side chains of a peptide may determine its effectiveness as an inhibitor (Marder *et al.* 1977). Methionine peptides are very effective competitors for the utilization of other peptides (Marder *et al.* 1977). Increased lag phases are observed because of competition at the level of transport, when these peptides are used as wort supplements with other peptides (Marder *et al.* 1977). The competing peptide is taken up by the cell and hydrolyzed by endogenous peptidases (Marder *et al.* 1977). After the concentration of the competing peptide is depleted by this process, the peptide containing the required amino acid can then enter and growth commences (Marder *et al.* 1977). These comments indicate that the composition of peptides is also an important determinant in the substrate specificity of the peptide permease system of the brewing yeast and in the initial uptake rates of peptides (Moneton *et al.* 1986; Nisbet and Payne, 1979).

However, single peptides are not necessary as a good source of nitrogen for growth as the amino acids that constituted them (Patterson and Ingledew, 1999). Therefore, growth on a particular amino acid could not be used to predict the growth characteristics on the

homologous di- or tripeptide (Patterson and Ingledew, 1999). For instance, the enhanced growth of yeast cells with arginine supplementation was not repeated with medium enrichment with $(Arg)_2$ and also poor cell division was observed (Patterson and Ingledew, 1999). The negative growth response on that dipeptide could be caused by the non transfer into the site of the peptidase activity inside the cell (Marder *et al.* 1977). Most of these peptidases are located either in intracellular organelles or in vacuoles and in the cytoplasm (Marder *et al.* 1977).

The inclusion of micromolar amounts of various amino acids in a medium that contains immobilized cells, either facilitates peptide transport or it inhibits their utilization (Island *et al*. 1987). Other studies have also confirmed this fermentation behaviour, where peptide utilization was affected by nitrogen supplementation of the medium with other sources than small peptides (Patterson and Ingledew, 1999). For instance, the uptake of Ala-Ala was promoted 3.5-fold by the addition of leucine, while addition of asparagine resulted in a decline of the peptide consumption rate (Patterson and Ingledew, 1999). The degree of growth enhancement and the length of the lag period prior to peptide utilization were found to be dependent on both the nature of the amino acid used and on the oligopeptide quality (Patterson and Ingledew, 1999). Although growth will be limited when the rate of supply of the nutrient peptide falls below that needed for optimal protein synthesis, it is difficult to understand why competition should lead to varied lag periods rather than varied growth rates and the simple competitive mechanism described by Marder *et al.* (1977) appears to be inadequate to explain the specificity in the observed growth inhibitions. The good growth peptides always exhibit a slight lag phase (5 to 7h), when their utilization is compared with individual amino acids (Marder *et al.* 1977).

Finally, it has been recognized that numerous nitrogenous materials from yeast are released into the wort during fermentation (Clapperton, 1971a). A great part of these compounds are oligopeptides, which are formed during fermentation and whilst some of these may be assimilated by the yeast, others will remain in the final beer contributing to its flavour and stability (Macwilliam and Clapperton, 1969). In addition, if the capacity of accumulated peptide residues is exceeded intracellularly then some of these might be released as deaminated derivatives (Woodward and Cirillo, 1977). The peptides remaining in the fermented wort are probably too large to be assimilated by the yeast given again the size restriction on peptide yeast preference (Calderbank *et al*. 1985).

1.7.2 The peptide transport system (PTR)

In brewing yeast, the absence of competition between the individual amino acids and simple peptides uptake, suggests that the peptide transport system is distinct from that of amino acids (Nisbet and Payne, 1979). It was also shown that di- and tripeptides share the same transport system (Nisbet and Payne, 1979). In addition, Patterson and Ingledew (1999), by using a synthetic medium with some amino acids and small peptides, found that no extracellular peptidase activity was produced, which suggests that small peptides are taken up by the yeast intact, via a specific peptide transport system. The induction of simple peptide transport is mediated by a specific metabolic sensor that triggers the rapid synthesis of an additional permease or catalytic activity that is capable of modifying the existing peptide transporter system (Island *et al.* 1987). In accordance with its role as a system providing nutrients in the form of peptides, it has been verified that the activity of this mechanism is modulated both by the quality of the nitrogen sources and the presence of amino acids included in the growth medium (Barnes *et al.* 1998). Initial peptide uptake rates were observed to be higher in cells that were grown on poor nitrogen sources, such as proline, than in cells grown on preferred nitrogenous materials, such as glutamine (Barnes *et al.* 1998). Based on this criterion, it has been proposed that the peptide transport system of yeasts falls under regulatory control of the nitrogen catabolite repression mechanism (Perry *et al*. 1994). Probably a similar inactivation occurred in the GAP1 system, may be induced in the peptide transport system, when yeast cells have been propagated in a medium with ammonium ions as the sole nitrogen source (Moneton *et al*. 1986). Nitrogen catabolic repression probably exerts a mild effect on peptide transport rate, whereas amino acids result in dramatic changes in initial oligopeptide uptake rates (Barnes *et al.* 1998).

The specific system mediating transport of intact small peptides across the plasma yeast membrane is an energy dependent process (Barnes *et al.* 1998). Once internalized, the peptides are rapidly hydrolyzed by intracellular peptidases and may then serve as a source of amino acids or nitrogen for growth (Barnes *et al.* 1998). In brewing yeast, the transport of peptides containing two or three residues is regulated by the protein products of at least three genes, *PTR1*, *PTR2* and *PTR3* (Barnes *et al.* 1998).

The Ptr2 protein for peptide transport across the plasma membrane is encoded by a 1,803bp DNA sequence and has a molecular weight of 68.1kDa (Perry *et al*. 1994). The penultimate amino-terminal amino acid residue is leucine, following only serine and

threonine in frequency at that position (Perry *et al*. 1994). Primary protein structure consists of a hydrophilic amino acid terminus spanning approximately 80 amino acids, an alternative pattern of hydrophobic and hydrophilic regions and a carboxy terminus spanning approximately 50 amino acids (Perry *et al*. 1994). The hydrophilic leader sequence and the alternating pattern of hydrophilic domains flanked by hydrophilic regions in the Ptr2 protein are typical of membrane-associated proteins involved in membrane transport (Perry *et al*. 1994). Without the existence of the *PTR1* gene, *PTR2* can not be expressed under any growth conditions studied (Alagramam *et al*. 1995).

Investigation of the *PTR2* gene indicated that the expression of this gene is regulated by amino acids in the medium (Perry *et al*. 1994). *PTR2* expression levels in amino acid-induced wild type cells were higher for cells grown on proline than for cells grown on ammonium ions, indicating that *PTR2* expression is regulated by the nitrogen source (Barnes *et al.* 1998).

The encoded product of the *PTR3* gene is a soluble protein, containing no hydrophobic domains that might span the plasma membrane (Barnes *et al.* 1998). It is relatively rich in serine and threonine, constituting 10% and 8% of these residues within its sequence, respectively. Previous studies had indicated that loss of Ptr3 function affected the peptide uptake sensitivity and affinity (Barnes *et al.* 1998). Ptr3 functions by modulating the activity of the dipeptide/tripeptide transport system in response to amino acids and demonstrate that Ptr3 is not required for catabolite repression of the PTR system, but Ptr3 might function by modulating *PTR2* expression. Deletion of *PTR3* does not seem to affect steady-state levels of *PTR1* expression. However, its deletion correlated with the loss of amino inducible peptide transport activity and also it was found that the observed attenuation in peptide transport also correlated with loss of amino acid-induced levels of steady-state *PTR2* expression. Therefore, it is very likely that Ptr3 functions as a facilitator of *PTR2* expression in response to amino acids found in the medium. These results also indicated that *PTR2* expression is determined by two separate systems: one that increases *PTR2* expression levels through *PTR3* in response to amino acid inducers and a second that functions independently of *PTR3* and is subject to nitrogen catabolite repression (Barnes *et al.* 1998).

1.7.3 Oligopeptides and other beer characteristics

Low molecular weight peptides are considered to act as foam negative effectors (Dale and Young, 1992). Addition of simple peptides to beer resulted in a decrease in the head retention of beer (Wainwright, 1978). A possible mechanism by which small peptides destabilize beer foam proposes that a limiting number of sites at the gas/liquid interface exist within foams, which are available for occupation by surface active molecules (Dale and Young, 1992). Small peptide molecules may occupy sites to the exclusion of polypeptides thereby resulting in a lower degree of foam stability than if all these sites were occupied by foam enhancing polypeptide molecules (Dale and Young, 1992). However, addition of tripeptides to beers showed that they did not have any significant effect on beer foam stability (Dale and Young, 1992).

Turbidity of colloidal nature that frequently appears in final beer, originates also from the oxidation of the present beer polyphenols, which form polymers that interact with the proline containing peptides and form a colloidal precipitate appearing as an irreversible haze (Outtrup 1989; Gorinstein *et al*. 1999).

1.8 Ammonia

Brewer's yeast is also capable of utilizing inorganic sources of nitrogen, such as ammonia. Ammonium ions are actively transported and readily assimilated by yeast. Ammonia uptake probably involves at least three permeases, Mep1p, Mep2p and Mep3p (ter Schure *et al*. 2000). Mep2p exhibits the highest affinity for ammonium ions, followed closely by Mep1p and finally by Mep3p (ter Schure *et al*. 2000). Growth on ammonium ions at concentrations higher than 20mM does not require any of the ammonia permeases (ter Schure *et al*. 2000).

In yeasts growing in the presence of ammonia, almost all the nitrogen is first assimilated into glutamine and glutamate (Holmes *et al.* 1989). Since these amino acids derive their alpha-amino nitrogen directly from ammonia, they are synthesized at a rate sufficient to provide the alpha-amino nitrogen required for yeast growth (Holmes *et al.* 1989). Other amino acids are formed by transamination reactions (Holmes *et al.* 1989). These amino acids then serve as precursors for the biosynthesis of other amino acids (Holmes *et al.* 1989). Glutamate and glutamine are therefore primary products of ammonium

assimilation and are key compounds in both nitrogen and carbon metabolism (Holmes *et al.* 1989).

The major route of ammonium assimilation in yeasts is into glutamate through the action of NADP-dependent glutamate dehydrogenase (GDH) (Lacerda *et al.* 1990). In this pathway, glutamate is generated via NADPH-GDH by coupling ammonia to α–ketoglutarate at the expense of one NADPH molecule (**Figure 14**) (Lacerda *et al.* 1990).

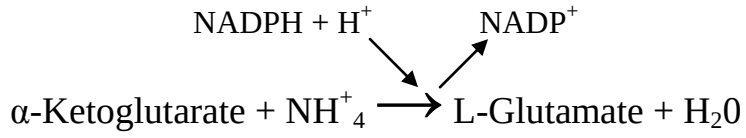


Figure 14: Glutamate formation from the coupling of ammonia and α-ketoglutarate

In the second pathway, glutamine is produced by glutamine synthetase (GS), which converts glutamate and ammonia into glutamine by consuming one ATP molecule (**Figure 15**) (ter Schure *et al*. 2000) and glutamate is synthesized by glutamate synthase (GOGAT), which converts one molecule of glutamine and one molecule of α–ketoglutarate into two molecules of glutamate at the expense of one NADH (**Figure 16**) (ter Schure *et al*. 2000).

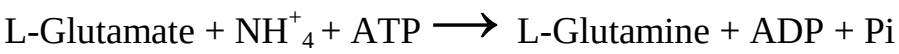


Figure 15: Conversion of glutamate and ammonia into glutamine

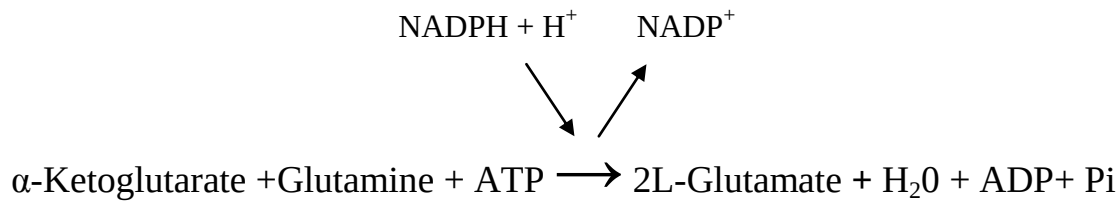


Figure 16: Glutamate production from α-ketoglutarate and glutamine

When glutamine synthetase is coupled with glutamate synthase, this glutamine pathway not only provides an alternative route to glutamate compared with the glutamate dehydrogenase reaction, but also represents a highly efficient process for yeasts to assimilate ammonia into alpha amino nitrogen. In addition, the glutamine pathway may also play an important role in yeast cell physiology such as the maintenance of citric acid cycle intermediates, cell growth and morphology (Magasanik, 1992). However, GS and GOGAT mediated assimilation of ammonia is clearly energetically less favourable than the glutamate dehydrogenase reaction because of ATP expenditure during the process (Magasanik, 1992).

The GDH and GS-GOGAT pathways are highly regulated and the particular route of ammonium assimilation adopted by yeasts will depend on various factors, not least the concentration of available ammonium ions and the intracellular amino acid pools.

Glutamate and glutamine can be also used as sole nitrogen sources (ter Schure *et al*. 2000). During growth on glutamate, GS produces glutamine using ammonia generated by NAD-GDH. When glutamine is the only nitrogen source in the growth medium, glutamate is synthesized by glutamate synthase or via NADPH-GDH, in the former case ammonia is produced by glutaminases which degrade glutamine to glutamate and ammonia (ter Schure *et al*. 2000).

Finally, it has to be discussed that ammonia inhibits the synthesis of numerous proteins involved in the assimilation of poorer nitrogen sources at the level of transcription (ter Schure *et al*. 2000). Growth on ammonia results in higher growth compared to growth on proline and this is caused by a higher metabolic nitrogen flux towards the synthesis of glutamate and glutamine. In other words, ammonia yields high intracellular concentrations that in turn trigger the transcription repression. During growth on ammonia these effects would originate from either the nitrogen flow or the nitrogen concentration. In the case of flux, higher metabolic fluxes would result in inactivation of

transcription (ter Schure *et al*. 2000). To be more precise, when continuous yeast cultures were used, it was shown that the expression of the *GAP1* gene was not changed by the ammonia flux but by the ammonia concentration (ter Schure *et al*. 2000). However, the increase in the ammonia ions levels resulted in an increase in the intracellular glutamine concentration and hence ammonia repression could be still generated indirectly via glutamine. Another conclusion that supports this suggestion is that *GAP1* expression in the continuous cultures correlated with the ammonia concentration whereas the intracellular glutamine concentration remained constant (ter Schure *et al*. 2000).

1.9 Yeast proteases

When wort is boiled before yeast pitching, the proteolytic enzymes from malt are destroyed and it is normally assumed that no further proteolysis occurs in the next stages of fermentation and maturation. However, this assumption is not correct because other proteolytic enzymes could escape from both damaged-dead and live yeast cells, especially under conditions of high gravity brewing where yeast cells are stressed for numerous reasons (Dreyer *et al*. 1983). The nature of the yeast strain affects the production and the proteolytic activity in fermenting wort and final beer (Dreyer *et al*. 1983). This may mirror differences in the cell membrane permeability from strain to strain, since it has been reported that excretion of intracellular proteases in the fermentation medium is possible from live yeast cells, when the medium contains proteins and polypeptides (Maddox and Hough, 1955).

In addition to yeast stresses induced under high concentrations of dissolved wort sugars, nitrogen starvation can also lead to protein degradation, mediated by the excretion of a sum of various proteinases. Four well-characterized proteinases have been isolated and examined from yeast and these are A, B (Hata *et al.* 1967, Lenney and Dalbec, 1967) and the carboxypeptidases Y, previously called proteinase C (Doi *et al*. 1967) and S.

Proteinase A is an endoproteinase with an acidic pH optimum (Saheki and Holzer, 1974). It is a glycoprotein of approximate size 60kDa and about 10% of which is contributed by carbohydrate (Saheki and Holzer, 1974). Proteinase B is a serine endoproteinase with a neutral pH optimum (Lenney and Dalbec, 1967). Estimates of the glycoprotein range from around 31kD to 44kD (Lenney and Dalbec, 1967). On the basis of the determination of 44kD, the carbohydrate content constitutes less than 0.5% of proteinase B (Ulane and Cabib, 1976).

Proteinase Y is also a serine based carboxypeptidase (Hasilik and Tanner, 1978). This glycoprotein is approximately 60kDa and $1/6^{th}$ of that is contributed by carbohydrate (Hasilik and Tanner, 1978). It is likely that the carbohydrate content consists of four asparagine-linked oligosaccharides of the general formula (*N*-acetyl-glucosamine)$_2$-(mannose)$_{13}$ (Hasilik and Tanner, 1978).

All these proteinases are located in the yeast vacuole and when the nitrogen wort content is not either rich in assimilable nitrogenous compounds or when the fermentation medium has reached nitrogen nutrient depletion, the proteinases are excreted into the external yeast environment (Wiemken *et al.* 1979). Their function is to attack larger peptides and proteins available in the wort and degrade them into simple peptides that can be used by the yeast as additional nitrogen sources (Dreyer *et al*. 1983). This phenomenon is considered to be a homeostatic mechanism in order for yeast cells to retain a high viability and also to ensure that the yeast metabolic activity and fermentation efficiency do not cease. Other reasons that lead to the excretion of these hydrolases in the fermentation medium include, yeast stresses developed under conditions of high gravity brewing and also when cells are reaching the death phase where their autolysis takes place and all these proteolytic enzymes are released in the fermenting wort (Dreyer, 1989).

1.10 Flavour compounds

Although, the major proportion of FAN is utilized for the synthesis of new cellular and enzymatic proteins of the yeast, it has been shown that there is a correlation between FAN and the formation of higher alcohols, esters, aldehydes and vicinal diketones, which affect and contribute drastically to final beer flavour characteristics (**Figure 17**) (Pierce, 1987).

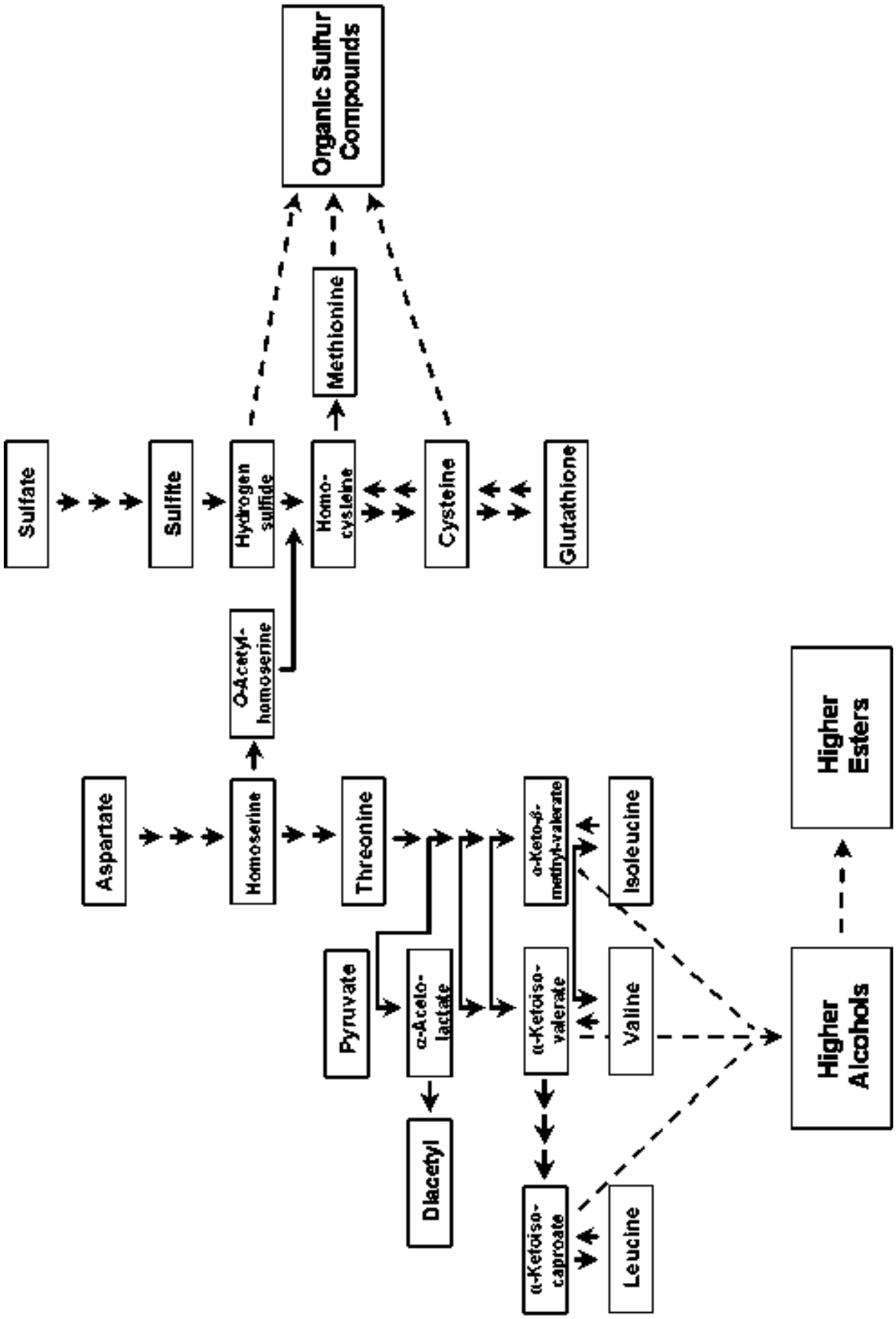


Figure 17: Amino acid metabolism and production of flavour compounds (Source: www.genome.ad.jp)

During the course of fermentation, the time course for the production of these flavour compounds has good correlation with yeast proliferation (Inoue and Kashihara, 1995). The latter shows that there is also a good correlation with the FAN assimilation rate (Inoue and Kashihara, 1995). At the end of yeast growth, all these processes cease and the levels of certain flavour compounds, including total diatecyl and acetaldehyde, decrease (Inoue and Kashihara, 1995). Quain and Duffield (1985) also reported an excellent linear relationship between yeast growth and total esters levels and higher alcohol synthesis. Thus, it is believed that yeast growth induced by ample FAN wort levels, is a better index of beer flavour and aroma than carbohydrate attenuation (Inoue and Kashihara, 1995). Based on that, it is expected that the relationships between flavour compounds and yeast growth/amino acid utilization can be applied so that high quality beer can be produced by controlling these two fermentation parameters (Inoue and Kashihara, 1995). High wort FAN causes negative effects on drinkability and beer stability (Pugh *et al.* 1997). Thus, the FAN composition of wort is consequently a rather important component of the complex system regulating the production of flavour active compounds formed by the yeast (Pugh *et al.* 1997).

1.10.1 Higher alcohols

The higher alcohols or fusel oils that are mainly found in final beer are n-propanol, isobutanol, 2-methyl-1-butanol and 3-methyl-1-butanol (Lewis and Young, 1995). The biosynthesis of these flavour compounds is very complex since they may be derived either by amino acid catabolism or via pyruvate carbohydrate metabolism (Lewis and Young, 1995). The catabolic process involves a pathway in which a keto-acid, derived by the deamination of an absorbed amino acid, is decarboxylated to the corresponding aldehyde and then reduced to the alcohol via an NAD-linked dehydrogenase (Lewis and Young, 1995). The higher alcohol formation from α–keto acids is dependent on the size and activity of the total metabolic system of nitrogen compounds, which is believed to be affected by the total uptake of nitrogenous wort compounds (Takahashi *et al.* 1997).

On the other hand, the anabolic process utilizes the same pathways as those involved in the amino acid biosynthesis. As in the catabolic route, the keto-acid intermediate is decarboxylated and the resultant aldehyde is eventually reduced to the alcohol (Lewis and Young, 1995).

In general, the catabolic route is followed at the early stages of fermentation, when the levels of assimilable yeast nitrogen are still high (Lewis and Young, 1995). Moving onto the later stages of fermentation, the anabolic pathway is activated for the formation of fusel oils, since the sources of utilizable nitrogen have been depleted (Lewis and Young, 1995). The production of higher alcohols in beer is strongly correlated with the yeast growth rate. Therefore, fermentation factors, such as amino acid supplementation, that might stimulate a high cell number during fermentation, are also indices for production of elevated levels of higher alcohols compared to the control fermentations.

The production of two very important alcohols, isobutanol and isoamyl, is correlated with the uptake of valine and leucine, respectively (Takahashi *et al.* 1997). These two fusel oils are not only produced when the incorporated amino acids valine and leucine are utilized via the Ehrlich's path, but their production is more strongly correlated with the total nitrogen material utilization (Takahashi *et al.* 1997).

1.10.2 Esters

Esters are also beer components that contribute to the flavour and aroma of the final product, best known for providing fruity characteristics (Lewis and Young, 1995). However, ester formation above its threshold admissible values, it is unpleasant for the organoleptical character of beer (Lewis and Young, 1995). The most important esters found in beer are: ethyl acetate, isoamyl acetate, isobutyl acetate and 2-phenylethyl acetate (Lewis and Young, 1995). Ethyl acetate production increases when the uptake rate of nitrogenous compounds decreases (Takahashi *et al.* 1997). The FAN wort content is indirectly involved with their formation since one of the two substrates responsible for their synthesis is a higher alcohol (apart from ethyl acetate, which derives from ethanol) (Lewis and Young, 1995).

Ester formation during fermentation does not follow the same pattern as higher alcohol and vicinal diketone synthesis (Inoue and Kashihara, 1995). Anderson and Kirsop (1974) believed that acetate ester formation is involved with amino acid metabolism and hence with yeast proliferation. Their hypothesis is based on the metabolism of acetyl coenzyme A (TCA cycle) during aerobic wort fermentations and during late anaerobic stages, acetyl coenzyme A forms acetate esters by alcoholysis with higher alcohols (Inoue and Kashihara, 1995). Esters derive from a reaction between an alcohol and a fatty acyl-CoA ester (Verstrepen *et al.* 2003). The enzyme family responsible for catalyzing this reaction

is known as alcohol acetyl transferases (Verstrepen *et al.* 2003). The acyl component of the activated fatty acid may be acetate produced by the action of pyruvate dehydrogenase (Verstrepen *et al.* 2003). In addition, acetate and longer chain acids may be activated directly by an acyl-CoA synthetase (Verstrepen *et al.* 2003).

The final levels of esters produced in beer are dependent on the wort gravity, the oxygen availability and also the incubation temperature (Brown and Hammond, 2003). Under conditions of high gravity brewing, especially when using adjunct worts, the FAN content decreases meaning that yeast multiplication is limited and the extra amounts of dissolved sugars are then converted into acetyl-CoA, which obviously promotes elevating ester production (Brown and Hammond, 2003). On the other hand, high initial levels of dissolved oxygen lead to lower ester production, since the excess of oxygen triggers increased amounts of cell biomass and thus the acetyl-CoA reservoirs are used in biosynthetic reactions (Brown and Hammond, 2003).

1.10.3 Vicinal diketones

Diacetyl and 2,3-pentanedione are carbonyl compounds formed during the course of fermentation. Their existence in final beer affects its flavour characteristics. Both of these compounds provide a "butterscotch" flavour and aroma in beers (Barton and Slaughter, 1992). Thus, the brewer should ensure that vicinal diketone levels in most mature beers should be lower than their threshold limits (Barton and Slaughter, 1992).

Formation of diacetyl and 2,3-pentanedione originates from the biosynthetic pathways of valine and isoleucine, respectively (Barton and Slaughter, 1992). The precursors of vicinal diketones, α–acetolactate and α–acetohydroxybutyrate, are intermediate metabolites in the above mentioned biosynthetic pathways (Barton and Slaughter, 1992). The release of these intermediates in the fermenting wort leads to their spontaneous oxidative decarboxylation, yielding the resultant vicinal diketones (Barton and Slaughter, 1992). Then diacetyl is reduced to acetoin and eventually to 2,3-butanediol. Similarly, 2,3-pentanedione is converted into its corresponding diol (Barton and Slaughter, 1992). The threshold values of these diols are relatively high and thus, the reducing steps of the vicinal diketones are very crucial in order to obtain a final product devoid by any unpleasant organoleptic properties (Barton and Slaughter, 1992).

Fermentation conditions that may promote increased cell biomass also stimulate increased amino acid biosynthesis to cover the need of cells for assimilable nitrogen for their metabolic activities (Barton and Slaughter, 1992). That will result in the production of elevated levels of the vicinal diketone precursors and hence greater production of diacetyl and 2,3-pentanedione (Barton and Slaughter, 1992).

The reduction of vicinal diketones during the stationary and death phase of fermentation and also during maturation of the green beer, needs the presence of ample yeast biomass in suspension (Barton and Slaughter, 1992). If the yeast is removed from the green beer prematurely then adequate diacetyl reduction does not occur, resulting in high undesirable levels in finished beer (Barton and Slaughter, 1992).

The valine consumption rate shows that there is a close relationship with acetolactate formation, the precursor of diacetyl (Inoue and Kashihara, 1995). Total diacetyl levels and final pH value can only be used qualitatively to detect abnormalities in the fermentation as their change during fermentation is not linear with yeast growth and amino acid consumption (Inoue and Kashihara, 1995).

Wort with high FAN content results in increased diacetyl production, extended lagering time and higher beer nitrogen residues (Pugh *et al.* 1997). On the other hand, worts with too low FAN content result in slower fermentations, reduced yeast crop and high diacetyl (Pugh *et al.* 1997). Ideally, wort should contain the least amount of FAN required for normal yeast growth and a minimum amount of diacetyl production (Pugh *et al.* 1997). This will result in shorter lagering times since less diacetyl is produced and the rate of diacetyl reduction is faster due to lower beer pH (Pugh *et al.* 1997). Thus, it can be outlined that valine could be used as a marker for FAN optimization (Pugh *et al.* 1997). The point that wort valine is totally used depicts the start of diacetyl production (Pugh *et al.* 1997).

1.10.4 Aldehydes

Aldehydes are also beer components that serve a significant role in the flavour of finished beers (Meilgaard and Peppard, 1986). Mainly, aldehydes provide beers with a "grassy" note and strong aftertaste especially above their threshold limits (Meilgaard and Peppard, 1986). The most well known aldehydes are acetaldehyde, propanal, 2-methyl butanal and pentanal (Meilgaard and Peppard, 1986). As it was discussed in the higher alcohols section (***1.10.1***), aldehydes are derived indirectly from the transport and

deamination of exogenous amino acids followed by decarboxylation of the resultant keto-acid (Meilgaard and Peppard, 1986). As with fusel oils and esters, the production rate of aldehydes is regulated by the yeast strain and the fermentation conditions e.g. high wort dissolved oxygen concentration, fermentation temperature and wort gravity (Meilgaard and Peppard, 1986).

Chapter 2: Materials and Methods

2.1 Shake flask fermentations

2.1.1 Maintenance of stock cultures

Yeast cultures of the genus *Saccharomyces cerevisiae* (ale strain No 70) taken from the ICBD yeast collection, were maintained on yeast peptone nutrient (PYN) agar (technical agar 4% (w/v)) slopes and stored at 4^{o}C. Sub-culturing of the cultures was carried out at two-month intervals onto freshly prepared agar PYN slopes, which were incubated for 72h at 25^{o}C and stored again at 4^{o}C.

2.1.2 Yeast identification

Identification of the selected yeast strain from the ICBD yeast collection was carried out prior to shake flask fermentations. The relevant yeast strain (No 70) was pitched in a 12^{o}Plato all malt wort and fermentation was conducted at 37^{o}C under static fermentation conditions. The yeast strain exhibited normal growth under these conditions, confirming the fact that the selected yeast strain was an ale strain of the genus *Saccharomyces cerevisiae*.

2.1.3 Preparation of inoculum cultures

Yeast cultures (ale No 70) were prepared by inoculating a loop of yeast cells from agar slopes, into six 10ml test tubes containing 5ml of sterile PYN broth. The PYN broth contained the following ingredients: peptone (bacteriological) (3.5g/l), yeast extract (3g/l), KH_2PO_4 (2g/l), $(NH_4)_2SO_4$ (1g/l), $MgSO_4.7H_2O$ (1g/l) and glucose (100g/l). Cultures were grown for 48h at 25^{o}C on an orbital shaker (SANYO Gallenkamp, Watford Herts, UK) at 150rpm. Then, the entire content of the six test tubes was added to a sterile 500ml conical flask containing 400ml of 12o Plato all malt wort. The flask was incubated for a further 48h at 25^{o}C on the orbital shaking incubator at the same speed, in order to attain log phase growth. After the incubation, the pitching volume needed for each fermentation was estimated by using the following equation (1):

$$\text{Pitching Volume} = \frac{\text{Live cells desired X Fermentation Volume}}{\text{Live cells counted}} \quad (1)$$

2.1.4 Wort preparation

Three different wort types were prepared for the conduct of the shake flask fermentations: 12°Plato and 20°Plato all malt and 20°Plato malt + 30% Glucose. All the brewer's worts used for the experiments were produced in the 2-hL pilot brewery facility of ICBD (**Figure 20**). For the production of worts, malted barley of the variety 'Optic' was used. For the production of the 12°Plato and 20°Plato all malt and 20°Plato malt + 30% Glucose worts, 34kg, 44kg, 25kg of malt were used, respectively. In addition, for the production of the high gravity adjunct wort, 8.1kg of glucose were utilized. The total wort volumes produced were 200L for the 12°Plato all malt wort, 150L for the 20°Plato all malt wort and 120L for the 20°Plato malt + 30% Glucose wort. The malt was provided by Bairds Malt Ltd**,** (Witham, UK) and it was stored at a constant temperature of 11°C, until used. The malt was milled using a two row M5 Brusier Fraser Agricultural mill (Fraser Agricultural Ltd, Inverurie, UK) and then mashed at a grist to aliquot ratio of 1 to 3 for 12°Plato wort and 1 to 2 for 20°Plato wort. The milled malt was placed in the mash tun and it was mashed at 65°C for 60min and then raised to 75°C and held constant for 1min for mashing off. Water at temperature 80-100°C was pumped to the 2hL lauter tun (Briggs of Burton, Burton on Trent, UK) to approximately 1cm above the false bottom. The mash was transferred to the lauter tun where it settled on the false bottom until the grist bed was formed. The extracted wort from the grist bed was circulated for 20min; until satisfactory wort clarity was achieved and then it was transferred in the kettle. As the spent grains still contained fermentable extract, the grist bed was sparged with water at 80°C. The rakes were used to enhance filtration. Once wort had reached a specific gravity between 1.5-2.5°Plato and the desired volume in the combined kettle/whirlpool was achieved, the sparging ceased. At this stage of production of the high gravity adjunct wort type, soluble glucose (adjunct) was added to the kettle in order to provide the fermentation medium with a higher percentage of fermentable sugars (extract). Hops were added in the form of pre-isomerised target pellets type 90 (11% α-acid) (The Wigan Hop Company Ltd., Edinburgh, UK) to give a potential finished beer

bitterness of 20 bitterness units (BU). The wort was boiled for 60min at an evaporation rate of 10% per hour. Kettle antifoam (Lipohop K^{TM}, Botanix Ltd, Kent, UK) was added at the beginning of each boil at dose rates of 2-5g/hL. After boiling, the wort was allowed to stand in the whirlpool for 25min. The wort was transferred through a paraflow heat exchange system (APV, West Sussex, UK) and collected at 4-8^{o}C in a 2hl fermenter (Briggs of Burton, Burton On Trent, UK). It was then cooled to a temperature range from 18-20°C. The cooled wort was finally transferred to two litre sterilized plastic bottles, frozen at -20°C and held until required for use.

2.1.5 Shaken fermentations

Three different wort types were used: 12^{o}Plato and 20^{o}Plato all malt and 20^{o}Plato malt + 30% Glucose, for the fermentations that took place in shake flasks. Wort was thawed then steam sterilized at 115^{o}C in an autoclave for 30min prior to fermentation. The yeast strain that was used for the pitching of the fermentation was an ale yeast (No 70) taken from the ICBD yeast collection. Fermentations were conducted at 20^{o}C in Erlenmeyer shake flasks sealed with highly porous thin pads, in an orbital shaking incubator at 150rpm. Samples for analysis were collected from the fermentations every 24h by using sterile glass pipettes.

2.2 Static fermentations

2.2.1 Yeast strains

The yeast strains employed for static fermentations were as follows: SCB5 (ale), SCB8 (ale), SCB3 (lager) and SCB4 (lager). All the ale and lager yeast cultures used for this study were strains of *Saccharomyces cerevisiae and Saccharomyces pastorianus*, respectively.

2.2.2 Yeast identification

Identification and verification of the various yeast strains provided by Scottish Courage Brewing Ltd for the conduction of the static fermentations was carried out prior to the

fermentation experiments. All the yeast strains were inoculated in a 12^{o}Plato all malt wort and fermentations were conducted at 37^{o}C under static fermentation conditions. The yeast strains that exhibited very poor growth under these conditions, were classified as yeast strains of the genus *Saccharomyces pastorianus* (lager yeast strains), while yeast strains that exhibited normal growth under these fermentations were identified as strains of the genus *Saccharomyces cerevisiae* (ale yeast strains).

2.2.3 Maintenance of stock cultures

Yeast cultures (SCB3, SCB4 and SCB8) were preserved on yeast and mould (YM) agar slopes. SCB5 cultures were preserved on 10^{o}Plato ale wort slopes (70% malt and 30% very high maltose syrup (VHM)) supplemented with 2% (w/v) Agar Technical (Oxoid Limited, Hampshire, UK). All the slopes were stored at 4^{o}C. Sub-culturing of the yeast strains was carried out at two-month intervals onto new agar slopes, which were incubated for 72h at 25^{o}C.

2.2.4 Propagation of yeasts

The steps followed for propagation of the various brewing yeast strains used, are illustrated in **Figure 18**. The contents of a yeast slope was inoculated into a 3L conical flask containing 1L of yeast and mould (YM) broth. The flask was incubated on an orbital shaker incubator (SANYO Gallenkamp, Watford Herts, UK) at speed of 150rpm, for 48h, at 18^{o}C. After the 48 hour incubation, 1L of YM broth was transferred into an 11.5L sterile Cornelius vessel and 8L of 70% malt + 30% VHM syrup wort, were also added. The vessel was continuously supplied via its inlet port with air flowing through a 0.45μm air filter connected to sterilized plastic tubing with a stainless steel sinter attached to the end of the tube, which was submerged in the fermenting liquid. The sinter was used for the production of very small size air bubbles so that yeast's oxygen absorption could be enhanced. The flow rate used was between 80-100L/h of air for 48h incubation at 18^{o}C. $ZnS0_4$ solution (22.5ml, 0.05μM, 200ppm with respect to zinc ions) and silicon-based antifoam agent (1ml) (Chemidex, Dormagen, Germany) were also added. The vessel was then transferred to a cold room at 4^{o}C to allow the yeast to settle for 24h.

Finally, the fermented wort was carefully decanted and the yeast slurry collected and transferred into a sterile container for pitching into wort.

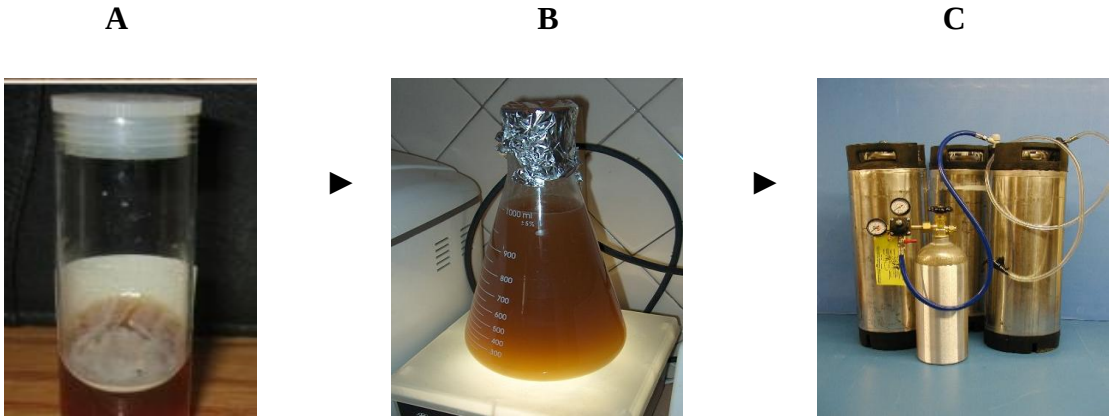


Figure 18: The yeast propagation steps

(**A:** Maintenance of yeast culture on the slope. **B:** Inoculation of the yeast slope contents into YM broth and growth on an orbital shaker. **C:** Continuous Cornelius vessel aeration system used for yeast propagation)

2.2.5 Determination of yeast pitching volume

The method that was used for the determination of pitching yeast concentration was described by Palmer (1969). The principle of this method is the quantification of yeast biomass in the slurry by determining the percent of yeast solids. First, an empty 50ml centrifuge tube was weighed. Immediately after mixing of the yeast slurry, approximately 50ml was dispensed into the tube. The tube was re-weighted and the weight of yeast slurry in the tube was measured by subtracting the weight of the tube full from the weight of the tube empty. The sample was centrifuged at 2,000g for 10min, the supernatant was carefully decanted and the tube re-weighed. The weight of yeast in the tube was recorded by using the following equation (2):

Weight of the tube after supernatant decanting – Weight of the tube empty (2)

The percent of solids (w/w) of the sample in the tube was calculated by using the following equation (3):

$$\text{Percent of solids} = \frac{\text{Weight of yeast X 100}}{\text{Weight of slurry}} \qquad (3)$$

The following step of the method was to determine the yeast fraction of the sample. Fifty to 60ml of mixed homogeneous slurry was transferred into a 50ml centrifuge tube. NaOH [2ml, 30% (w/v)] was added and the sample mixed vigorously by shaking. Immediately, the alkali treated yeast was decanted to the 50ml mark on a graduated centrifuge tube and the mixture centrifuged at 2,000g for 10min. After centrifugation, the yeast under the darkened barm beer could be seen to have been separated into three different layers:

A (Top): Dark brown/green protein trub
B (Middle): Creamy coloured yeast
C (Bottom): Green/granular hop trub (This layer was not always present)

Following the alkali treatment, the total alkali solids volume was recorded (**A**+**B**+**C**). The individual volumes of protein trub (**A**), yeast (**B**) and hop trub (**C**) (if it was present) were also recorded and the yeast fraction was calculated by using the equation (4):

$$\mathbf{Y_F} = \frac{B}{\text{Total Alkali Solids (A+B+C)}} \qquad (4)$$

Finally, the total percent of solids determined before, was multiplied by Y_F, the yeast fraction determined by alkali treatment in order to determine the percentage of yeast solids (equation (5)):

$$\%\ \text{yeast solids} = Y_F \text{ X } \%\ \text{total solids} \qquad (5)$$

The volume of yeast slurry for pitching was calculated with the following equation (6):

$$\text{Pitching Volume} = \frac{\text{Pitching rate X Total fermentation volume}}{\%\ \text{yeast solids X Viability fraction}} \qquad (6)$$

2.2.6 Wort preparation

The wort type (15^{o}Plato, 70% malt and 30% VHM syrup) used for the conduct of all the static fermentations, was produced in the 2hl ICBD brewery facility (**Figure 20**). For the production of the high gravity adjunct wort, 28kg of lager malt and 11.6kg of very high maltose syrup were utilized. The total wort volume produced was 180L. This particular wort type was produced employing the procedures and conditions already described in section ***2.1.4***.

2.2.7 Fermentations

Wort obtained from the same malt batch was used for all the static fermentations being carried out. Wort was thawed, steam sterilized at 115^{o}C in an autoclave for 30min and oxygenated before being pitched with yeast. Oxygenation of wort employed 100% oxygen flowing through a 0.45μm air filter connected to a sterilized rubber tubing with a stainless steel sinter attached to the end of the tube. Each wort sample was oxygenated according to the yeast's oxygen requirements in terms of oxygen flow rate and time (e.g 15ppm DO for 3min, for the lager strain SC3).

The fermentations took place in sterilized 2L cylinders (**Figure 19A**) sealed with a rubber bung and a fermentation lock filled with 70% ethanol. $ZnS0_4$ solution (1.5ml, 0.05μM, 200ppm with respect to zinc ions) and 40μl of a silicon based antifoam agent were added.

Aseptic techniques were observed at all times of sampling by using sterile glass pipettes (**Figure 19B**). The temperature of the incubation room was 15^{o}C for lager and 18^{o}C for ale fermentations. All fermentations were carried out in triplicate.

A **B**

Figure 19: A) 2L cylinders used for static fermentations, **B)** Wort sampling

Figure 20: The 2hL ICBD pilot brewery

2.3 Experimental wort and beer analysis

2.3.1 Determination of cell number and viability

Cell count of wort samples taken during the fermentations were determined by using an Improved Neubauer Haemocytometer (Weber Scientific Int., Middlesex, UK) at x400 magnification on a light microscope. Yeast viability was determined by the methylene violet staining method described by Smart *et al*. (1999). The sample was diluted with an equal volume of methylene violet reagent consisting of 10mg methylene violet dissolved in 10ml distilled water. Two grams of sodium citrate dehydrate were added, stirred until dissolved, filtered and made up to 100ml with distilled water. Dead cells stained purple and the percentage of colourless cells was used as a measure of viability. The method assumes that only the viable cells reduce methylene violet to its colourless form. Assays were carried out in triplicate and results reported as mean values ± S.D.

2.3.2 Biomass determination

Ten ml aliquot of fermenting wort was centrifuged at 5,000g for 5min. The supernatant was used for other measurements, while the residual pellet was washed twice with ammonium sulphate solution (10ml, 5M) and once with distilled water (10ml). The final pellet was transferred with 2ml of 96% ethanol to a tared aluminium dish. The dish was dried in an oven for 24 hours at 105^{o}C and allowed to cool in a desiccator for 30min. It was weighed immediately. The results are the average values of triplicate determination of each fermentation.

2.3.3 Determination of pH

The pH was monitored using a BDH Gelphas pH electrode attached to a Hamma pH meter (Merck/BDH) fitted with temperature compensation. The results represent the mean of three determinations, one from each triplicate for each fermentation.

2.3.4 Determination of specific gravity

Samples were centrifuged for 5min at 5,000g and the specific gravity of the supernatants was measured using a DMA 46 calculating digital density meter (PAAR Scientific Ltd., London). The results are the mean value of three determinations, one from each fermentation performed in triplicate.

2.3.5 Determination of total wet and dry yeast biomass

When fermentations were complete, the 2L cylinders were transferred to 4^{o}C for 24h, to allow the yeast cells to settle. After the 24h incubation, cylinders were removed from the 4^{o}C room and the fermented wort was removed from the cylinders by using a tubing system connected to a peristaltic pump. The pump was used so that the yeast crop that was well flocculated in the bottom of the cylinders was not disturbed. A small volume of fermented wort was left at the bottom (100ml) and a sterile glass rod was used in order to mix the yeast crop. The yeast crop was decanted into a swinging centrifuge bucket of known weight. The sample was then centrifuged at 5,000g for 10min and the supernatant decanted. The bucket was re-weighed and the weight of the total wet yeast crop was calculated by subtracting the weight of the bucket after decanting the supernatant from the weight of the empty bucket.

In order to determine the total dry yeast crop, 1.5g of the wet yeast crop was collected in a pre-weighed aluminium tray and then placed in an oven at 105^{o}C for 7 days. After the seven days incubation, the tray with the dried yeast sample was re-weighed and the total dry yeast crop was calculated with the following equation (7):

$$\text{Total dry yeast crop} = \frac{\text{Total wet yeast crop X Weight of the dried yeast sample}}{\text{Weight of the yeast sample used for drying}} \quad (7)$$

2.3.6 Determination of free amino nitrogen (FAN)

FAN was determined using the method described by Lie (1973). Samples were diluted with distilled water 100-fold for wort and 50-fold for beer. The diluted samples (2ml) were transferred to test tubes and 1ml of ninhydrin coloured reagent added (disodium

hydrogen phosphate, 100g; potassium dihydrogen phosphate, 60g; ninhydrin, 5g; fructose 3g in 1L of distilled water). The test tubes were covered with glass marbles so that evaporation losses were minimized and the test tubes heated for 16min in a boiling water bath. Ninhydrin, at this temperature, decarboxylates and deaminates amino acids and small peptides and produces a purple colour, which absorbs light maximally at 550-570nm. After heating, the samples were transferred to a water bath at 20^{o}C to allow cooling for 20min. When cool, 5ml of dilution reagent (potassium iodate, 2g; distilled water 600ml and 400ml of 96% (v/v) ethanol) was added and absorbance read at 570 nm using an Ultrospec II spectrophotometer, against a water blank and a glycine standard. The results in mg/L FAN were calculated by the ratio of the absorbance of the test solution to the glycine standard, multiplied by the dilution factor. From each triplicate fermentation, the samples were assayed in triplicate, and the results were consequently the mean values of the triplicates.

2.3.7 Determination of individual wort amino acids

Quantification of individual wort amino acids was achieved by gradient elution High Performance Liquid Chromatography (HPLC) using dansyl chloride as the fluorescence indicator (Mackey and Beck, 1982; Wiedmeier *et al.* 1982, instruction manuals of Jasco and Gilson). All amino acids were fully resolved by this method.

Fermentation samples were centrifuged to remove any remaining yeast cells and the supernatants filtered through 0.2μm syringe filters and diluted with sodium carbonate (0.2M; pH 9.7), prior to the reaction with the dye.

Samples were then dansylated by adding 20μl of sample/standard, 20μl of internal standard (norvanile 0.5mM) in a 1.5ml screw-capped reaction vial, sodium carbonate (200μl; 0.2M; pH 9.7) and 200μl dansyl chloride (5mg in 1ml acetone). The vials were sealed, placed in a sonicator (Decon FS Frequency Sweep, Ultrasonics Ltd.) for 5min and finally stored at room temperature overnight. After incubation, the volume of the samples was diluted up to 1ml with the addition of Millipore water. All samples were filtered through 0.2μm syringe filters post dansylation.

The samples were bound to an HPLC column and the individual amino acids were eluted using an eluent gradient. The column exclusion solution passed through a continuous fluorescent detector, which recorded the dansyl chloride concentration, which was

relative to the concentration of each amino acid. The system was calibrated with a calibration Sigma amino acid standard mixture AA-S-18 (Sigma, St Louis, USA) containing known amounts of the amino acid spectrum.

The internal standard solution was prepared as follows: a norvaline solution (100mM) was prepared using 0.1M HCl solution as solvent and the mixture was diluted 1:200 to give a 0.5mM final solution. This was divided into aliquots, which were kept frozen.

The calibration standard was prepared as follows: 100mM solutions of cysteic acid, cysteine, hydroxyproline, homoserine, taurine, ornithine, 40mM norleucine and 50mM methionine were prepared in 0.1M HCl. Twenty five μl of the 100mM, 62.5μl of the 40mM and 50μl of the 50mM solutions were added to 1ml of the Sigma amino acid standard solution AA-S-18. This contained amino acids at 2.5mM, apart from cysteine, which was contained at 1.25mM. The volume was made up to 5ml by adding 0.1M HCl. This produced a standard solution containing 0.5mM of each amino acid except cysteine at 0.25mM.

Eluents were produced by first preparing 20mM di-sodium hydrogen orthophoshate solution (5.678g were dissolved in 1.9L of distilled water). The pH of the solution was buffered to 6.19 and the volume was made up to 2L in a volumetric flask.

Eluent A consisted of the di-sodium hydrogen orthophosphate solution with 5% acetonitrile added. Eluent B consisted of a 2:3 solution of di-sodium hydrogen orthophosphate solution and acetonitrile.

The instrumentation used included: a Gilson 231 autosampler with a 401 dilutor, a Rheodyne 7010 injector with a 20μl loop, a Gilson 306 pump with a 5SC pump head, a Gilson 302 pump with a 5SC pump head, a Gilson 802 Manometric controller, a Gilson 811C dynamic mixer, a Jasco FP1520 Fluorescence detector, a Gilson 715 data handling package and a Phenomenex Degassex DG4400 degassing unit. The column was a Phenophere Next 5μm C18 150 x 4mm (Phenomenex UK Ltd.) placed in an oven at 27° C.

The pump program that was used for the production of the eluent gradient is shown in **Table 3**. The increasing amount of Eluent B removed the amino acids from the column.

Table 3: Eluent gradient program used for amino acids

Time (minutes)	% Eluent A	% Eluent B
0	93	7
35	87	13
45	87	13
50	80	20
60	72	28
70	64	36
80	56	44
85	52	48
95	44	56
100	40	60
105	30	70
110	30	70
110.1	0	100
115	0	100
115.1	93	7

The flow rate used was 1.5ml/minute and the run time was 155min. All amino acids were detected at 10pM injected. The retention times of the individual amino acids are shown in **Table 4**.

Table 4: Amino acid retention times

Amino Acid	**Time (minutes)**
Aspartic Acid	22.50
Glutamine & Glutamic Acid	25.09
Hydroxyproline	43.83
Homoserine	52.76
Serine	53.55
Arginine	54.02
Threonine	56.71
Glycine	57.81
Alanine	59.89
Taurine	63.67
Proline	64.12
Valine	69.07
Norvaline (internal standard)	71.55
Methionine	72.39
Isoleucine	75.33
Leucine	76.11
Norleucine	78.54
Phenylalanine	79.95
Cysteine	85.73
Ornithine	100.20
Lysine	100.94
Histidine	101.42
Tyrosine	109.71

2.3.8 Determination of ammonia

A spectrophotometric assay was used for ammonia determinations (Jansen *et al.* 1985). All samples were diluted according to the original gravity of the wort (e.g 15^{o}Plato wort, 1:15 dilution) with millipore water prior to analysis. The calibration standard was made from ammonium chloride and distilled water. The stock solution contained approximately 5-6ppm of NH_4^+. Dilutions of this stock standard were used to produce a calibration curve linear to 6ppm.

The samples for analysis were prepared as follows: 1.5ml of diluted sample or standards and potassium ferrocyanide (225µl; 10.6% (v/v)) was added and vortexed. Zinc acetate (225µl; 22% (v/v)) were also added, mixed well and filtered through a 1µm syringe filter. Afterwards, to 1ml of filtrate, 200µl of caustic mix (2% NaOH; 18% sodium citrate and 0.4% dichloroisocyanurate) were added. Two hundred µl of colour reagent (3.5% phenol, 0.04% sodium nitroprusside) were also added.

Finally, the mixture was heated at 65°C for two minutes and then left to cool. The absorbance of each sample was measured at 665nm against a distilled water blank, by using a Ultrospec II visible spectrophotometer. Amino acids did not pose an interference problem, probably due to the short oxidation step.

2.3.9 Oligopeptide determination

2.3.9.1 Sample preparation

Samples were centrifuged at 13,000g for 15min. and trichloroacetic acid (TCA) (3ml; 40%) added to the supernatant (3ml) and samples placed in ice for 60min. Samples were re-centrifuged at 13,000g for 10min and the resulting supernatants were filtered through 0.2µm syringe filters. The filtrates were stored at 4^{o}C for 3 days to enhance further protein precipitation and once again the supernatants were collected. Samples were then frozen awaiting future analysis.

2.3.9.2 Vacuum filtration

The cellulose ester ultra-filtration membranes (NBS Biologicals Ltd, Huntingdon, UK), used for the isolation of small oligopeptides, had a molecular weight exclusion of 500 Daltons. The membranes were washed with 20ml of distilled water prior to use and then were carefully placed at the bottom of the funnel. Small quantities of the samples (approximately 50µl) were pipetted in the middle of the membrane trying to avoid samples being absorbed around the perimeter. After 300µl of sample had been filtered, the membrane was blocked and no further sample was able to permeate.

2.3.9.3 Acid hydrolysis

The oligopeptide determination method is a modified version of the technique described by Dale *et al.* (1989). Each sample (100µl) was placed in a pyrex glass tube with cold performic acid (200µl). Each tube was sonicated for 3min and stored at 4^{o}C overnight. Sodium metabisulphite (50mg) and 7.5M hydrochloric acid (800µl) were added to each tube, which was sonicated again for 15min, then placed into a heating block (110°C; 24 hours). After the incubation period, the samples were each diluted to 5ml with deionised water and filtered (0.45µm). Each filtrate (4ml) was evaporated to dryness using a rotary evaporator. Samples were then re-suspended in sodium carbonate (800µl; 0.2M; pH 9.7).

2.3.9.4 Alkaline hydrolysis

Alkaline hydrolysis was used to recover tryptophan, which was destroyed by acid treatment. Each sample (100µl) was placed in a pyrex glass tube. Tubes were cooled in ice and 4.2N NaOH (5ml) was added. Mixtures were then placed in a heating block and incubated for 24h at 110°C. The following day, 6N HCl (5ml) was added and the contents diluted to 12ml with deionised water and filtered (0.45µm). Each filtrate (10ml) was evaporated to dryness. Finally, samples were re-suspended in sodium carbonate (2ml; 0.2M; pH 9.7).

2.3.9.5 HPLC analysis

HPLC analysis of hydrolysis products was carried out. Unhydrolyzed samples were also analysed to calculate the concentration of individual wort amino acids. Oligopeptide levels were estimated by subtracting the values obtained with the unhydrolyzed samples from the hydrolyzed samples.

2.3.10 Determination of protease activity

General protease activity was measured with a modified version of the method described by Mochaba *et al.* (1993). The principle of this method is that following treatment with proteases, resorufin-labeled peptides are released from resorufin labeled casein and they cannot precipitate with trichloroacetic acid (TCA), thus their concentration in the supernatant is equivalent to the proteolytic activity.

Each fermentation sample was centrifuged at 13,000g for 15min and the resultant supernatants were filtered through a 0.45µm syringe filter. For each assay, citrate-phosphate buffer (75µl; pH 5.3) and cell free fermentation samples (75µl) were added to an aliquot of universal protease substrate (50µl; resorufin labeled casein) (Roche Diagnostics Ltd, East Sussex, UK). After mixing, tubes were incubated at 37^{o}C for 2h. Five percent TCA (480µl) was then added to each tube to halt proteolytic activity and the tubes incubated for a further 10min at 37^{o}C. Each tube was then centrifuged at 10,000g for 10min and the supernatant (400µl) was added to Tris-HCl buffer (600µl; 0.5M; pH 8.8). The absorbance was measured at 574nm against the blank, which contained citrate-phosphate buffer (75µl; pH 5.3), universal protease substrate (50µl; resorufin labeled casein) and instead of containing cell free fermentation samples, contained 75µl distilled water. The colour changed within a short time from bluish-violet to red, if sufficient protease activity was present in the fermentation cell free samples in comparison to the blank sample, which contained distilled water.

The assay was calibrated with yeast proteinase A (PrA) (Sigma, St Louis, USA). The calibration curve was produced by preparing solutions of PrA from 0 Units/ml to 3.75 Units/ml (**Figure 21**).

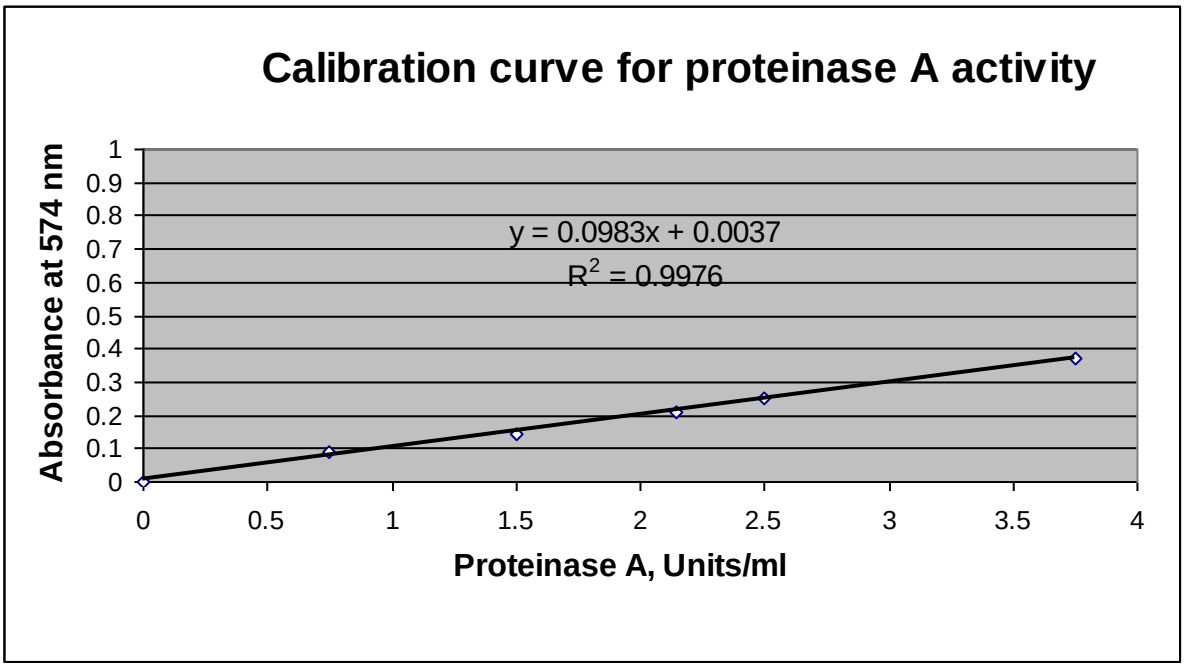


Figure 21: The calibration curve produced with PrA samples

2.3.11 Determination of carbohydrates by HPLC

Separation of carbohydrates was achieved by high performance anion exchange (HPAE). At high pH, carbohydrates are partially ionized and can therefore be separated by anion exchange. Detection was continuous by a pulsed amperometric detector (PAD) and involved measuring the electrical current generated by the oxidation of carbohydrates by the high pH of the eluent at the surface of a gold electrode.

Fermentation samples, prior to analysis were diluted with Millipore water, depending on their specific gravity:

Gravity	Dilution factor
1.050 – 1.046	1: 200
1.031 – 1.034	1: 100
1.018 – 1.011	1: 50

300µl of diluted sample or standard were mixed with 60µl of internal standard (600 ppm Cellobiose) and 20µl injected to an HPLC system. Samples were bound to an HPLC column and eluted using an eluent gradient. Each sample included an internal standard (600ppm Cellobiose), and the system was calibrated with a calibration standard containing known amounts of sugars.

The instrumentation included: a Pulsed Electrochemical Detector with a gold electrode (Dionex), a Gilson 302 pump, a Gilson 305 pump, a Gilson 802 Manometric Module, a Gilson 811B Dynamic Mixer, a Hewlett Packard 1050 Autoinjector, a Dionex eluent de-gas module, a Hewlett Packard Chemstation data handling system (HP3365) and a Dionex HPLC System. The Gilson HPLC system provided the gradient pumping, sample injection and column separation of components.

The Dionex system was used for post-column addition of 500mM NaOH at 0.6ml/minute. Mixing of the column effluent and the post-column addition was by means of a T-piece followed by a delay coil placed in a pre-detector. The eluents for the Gilson system were left at room pressure, while the Dionex system eluent was pressurized under helium. The column was a Dionex Carbopac PA-1 4 x 250mm column, used with a Dionex Carbopac PA-1 4 x 50mm guard column.

The calibration standard was prepared by addition of 10mg glucose, fructose, sucrose, cellobiose, maltotriose and 50mg maltose to a 100ml volumetric flask and diluting to volume with distilled water. This produced a 100mg/L solution of each sugar, except for maltose, which was 500mg/L.

The eluents comprised: Eluent A, millipore grade water and Eluent B, 500mM sodium hydroxide. Both eluents were sparged with helium for 15min prior to use. To each eluent, low carbonate concentrated NaOH was added (22ml, 47.3% w/v). The eluent gradient program is shown in **Table 5**, whilst the retention times of the sugars are shown in **Table 6.**

Table 5: Eluent gradient program used for sugars

Time (minutes)	**% A**	**% B**
0	80	20
13	70	30
20	50	50
25	25	75
28	0	100
31	0	100
31.1	80	20

Table 6: Retention times of sugars

Sugar	Retention Time (minutes)
Glucose	3.96
Fructose	4.58
Sucrose	7.33
Cellobiose internal standard)	8.90
Maltose	12.06
Maltotriose	22.60

Results are expressed in mg/litre, the original dry weight in 1ml was multiplied by 1000 to give the weight per litre. The results were divided by this weight to give the percentages of the individual sugars present. This was used to calculate the weights of carbohydrate in the original samples.

2.3.12 Determination of ethanol

Ethanol concentration was measured using a Chrompack CP9000 gas chromatograph with a split/splitless injector, flame ionization detector (FID) and a Hitachi-Merck D2000 integrator. The column was a CP SIL 5CB (10m X 0.32mm, 1.2μm film thickness) preceded by a 1m fused guard column. The temperature parameters used were: injector 180^{o}C, oven 100^{o}C and detector 200^{o}C. The flow rates of gases were: helium CG-2, 1.5ml/min (carrier gas), hydrogen, 35ml/min, air, 350 ml/min and auxiliary (OFN-oxygen free nitrogen), 32ml/min. The split ratio was 133:1. For calibration, a mixture of equal volumes of the ethanol standard (5% (v/v) ethanol [99.9%]) and the internal standard (5% (v/v) n-butanol [>99%]) was used. This method allows determination of ethanol concentrations up to 5% ABV by direct sample injection. Samples with higher than 5% ABV content were diluted with distilled water. The sample/diluted sample was mixed with an equal volume of internal standard and 1μl of mixture injected for ethanol analysis. From each fermentation (triplicate), the sample was assayed in duplicate; therefore the results represent the mean value of six determinations.

2.3.13 Determination of volatile compounds

Headspace analysis of esters (ethyl acetate, ethyl butyrate, ethyl hexanoate and isoamyl acetate), higher alcohols (propan-1-ol, isobutanol, 2-methyl butanol and 3-methyl butanol) and vicinal diketones (2,3-butanedione and 2,3-pentanedione) were monitored using a Hewlett Packard 5890 Series II GC with split/splitless injector, Electron Capture Detector (ECD) for vicinal diketones and Flame Ionization Detector (FID) for all the other flavour compounds, Hewlett Packard 19395 A Headspace Autosampler with 1ml injection loop and Hewlett Packard Chemstation Data Handling System. The column used was a Chrompack CP-Wax-57-CB (60m X 0.25μm, 0.40μm film thickness). The flow rates of gases were: helium (carrier gas), 45ml/min, hydrogen, 35ml/min, air, 350ml/min and auxiliary (nitrogen), 35ml/min. The temperature of injector and detector were 160 and 180^{o}C, respectively. The column temperature program was: 43^{o}C for 2min, raised to 92^{o}C at 1.8^{o}C/min, 92 to 180^{o}C at 30^{o}C/min and held at 180^{o}C for 4min. The internal standard used where 3-heptanone (200mg/L) and 2,3-hexanedione (18.1mg/L). Samples were prepared in a Chromacol 20ml vial to which was added 2g sodium chloride AnalaR (Sigma Ultra, Sigma St. Louis, USA), 5ml sample and 50μl of internal standard solution. The vial was capped, crimped and frozen until analysis. Prior to injection, each sample was preheated at 60^{o}C for 90min. Unheated samples were also measured to calculate the concentration of the VDK precursors acetolactate and acetohydroxybutyrate. These measurements were conducted in the final samples of supplemented fermentations with ammonia and amino acids. Precursor levels were estimated by subtracting the values obtained from the heated samples from the unheated samples.

At the end of the fermentations, three samples were collected from each fermentation and the values given in the results are the means from the replicate fermentations ± S.D.

2.4 Re-pitching of static fermentations

The yeast crop that was produced during the course of static fermentations, was collected and it was used for pitching of a subsequent series of static fermentations. The procedure used for the collection of the yeast slurry and determination of the pitching volume are described in the determination of total wet and dry yeast biomass and the determination of yeast pitching volume sections, respectively.

2.5 Amino acid supplementations

Supplementation of wort (15°Plato containing 30% VHM syrup) was carried out with two and five times the natural wort concentration of L-lysine, L-methionine, L-arginine and ammonia. All the amino acids were added into the 2L cylinders in dry form, after being carefully weighed. Ammonia was added in liquid form. One liter of wort and a magnetic flea were then added and the cylinder placed on a stirrer. The mixture was mixed until the amino acids were completely dissolved (approximately 30min). A further 500ml of wort was added and the fermentation oxygenated according to the oxygen requirements of the yeast strain. Finally, the appropriate yeast concentration was added to start the fermentation. Aseptic techniques were used during the preparation of the fermentation medium.

From this series of experiments, it was found that some of the amino acids had an inhibitory effect and some a beneficial effect on the yeast metabolic and fermentative activity. Thus, a new series of amino acid and ammonia supplementations was conducted, in order to investigate any synergistic effect between the wort nitrogenous materials and to discover any key nitrogen compounds that stimulate enhanced fermentation efficiency. This time, the supplementations were carried out with only twice the natural wort concentration for all the amino acid wort spectrum and ammonia. Any unfermented wort pH changes, induced by the supplementation of amino acids and ammonia, were adjusted with the addition of either HCl or NaOH solution. The following tables (**7A and 7B**) illustrate the number of possible combinations of amino acids and ammonia mixtures that were used. For the fulfillment of the experimental design of this trial, the statistical program Minitab (Minitab 14 statistical software, Minitab Ltd, Coventry, UK) was used.

Table 7A: The 25 different possible amino acid and ammonia supplemented fermentation combinations resulted after statistical experimental design analysis with Minitab. The amino acid and ammonia illustrated concentrations are in mmoles/L.

Wort A.A.	**Glu**	**Asn**	**Gln**	**Ser**	**Arg**	**Thr**	**Lys**	**Asp**	**NH_3**	**Val**
Ferm 1	0.9	0.56	0.32	0.94	1.18	0.84	1.2	0.39	1.78	0.96
Ferm 2	0.9	0.56	0.32	0.47	0.59	0.84	1.2	0.39	1.78	1.92
Ferm 3	0.9	1.12	0.32	0.94	0.59	0.42	0.6	0.39	3.58	0.96
Ferm 4	0.45	1.12	0.16	0.47	1.18	0.84	0.6	0.39	3.58	1.92
Ferm 5	0.45	0.56	0.16	0.47	1.18	0.42	1.2	0.39	1.78	1.92
Ferm 6	0.9	1.12	0.16	0.94	0.59	0.84	1.2	0.78	3.58	1.92
Ferm 7	0.9	1.12	0.16	0.47	1.18	0.84	0.6	0.78	1.78	1.92
Ferm 8	0.9	1.12	0.16	0.47	0.59	0.42	1.2	0.39	3.58	0.96
Ferm 9	0.45	1.12	0.16	0.94	1.18	0.84	1.2	0.78	1.78	0.96
Ferm 10	0.9	0.56	0.16	0.94	1.18	0.42	1.2	0.39	3.58	1.92
Ferm 11	0.45	1.12	0.32	0.47	1.18	0.42	1.2	0.78	3.58	1.92
Ferm 12	0.45	0.56	0.32	0.47	1.18	0.42	0.6	0.78	3.58	0.96
Ferm 13	0.45	1.12	0.32	0.47	0.59	0.84	1.2	0.39	3.58	0.96
Ferm 14	0.9	0.56	0.16	0.94	1.18	0.42	0.6	0.78	3.58	0.96
Ferm 15	0.45	1.12	0.16	0.94	0.59	0.42	1.2	0.78	1.78	0.96
Ferm 16	0.45	0.56	0.16	0.94	0.59	0.84	0.6	0.39	3.58	1.92

Wort A.A.	**Glu**	**Asn**	**Gln**	**Ser**	**Arg**	**Thr**	**Lys**	**Asp**	**NH_3**	**Val**
Ferm 17	0.9	0.56	0.16	0.47	0.59	0.84	0.6	0.78	1.78	0.96
Ferm 18	0.45	1.12	0.32	0.94	1.18	0.84	0.6	0.39	1.78	0.96
Ferm 19	0.45	0.56	0.32	0.94	0.59	0.42	1.2	0.78	1.78	1.92
Ferm 20	0.9	1.12	0.32	0.47	0.59	0.42	0.6	0.78	1.78	1.92
Ferm 21	0.9	0.56	0.32	0.47	1.18	0.84	1.2	0.78	3.58	0.96
Ferm 22	0.675	0.84	0.24	0.705	0.885	0.63	0.9	0.585	2.68	1.44
Ferm 23	0.9	1.12	0.32	0.94	1.18	0.42	0.6	0.39	1.78	1.92
Ferm 24	0.45	0.56	0.32	0.94	0.59	0.84	0.6	0.78	3.58	1.92
Ferm 25	0.45	0.56	0.16	0.47	0.59	0.42	0.6	0.39	1.78	0.96

Table 7B: The 25 different possible amino acid and ammonia supplemented fermentation combinations resulted after statistical experimental design analysis with Minitab. The amino acid and ammonia illustrated concentrations are in mmoles/L.

Wort A.A.	**Met**	**Ile**	**Leu**	**His**	**Gly**	**Ala**	**Trp**	**Phe**	**Tyr**	**Pro**
Ferm 1	0.2	1.14	1.11	0.68	0.6	1.2	0.42	1.8	0.62	3.7
Ferm 2	0.4	0.57	2.22	0.34	1.2	2.4	0.42	1.8	1.24	3.7
Ferm 3	0.4	0.57	1.11	0.68	1.2	1.2	0.21	1.8	1.24	3.7
Ferm 4	0.2	1.14	1.11	0.68	1.2	2.4	0.42	1.8	0.62	3.7
Ferm 5	0.4	0.57	1.11	0.68	1.2	1.2	0.42	0.9	1.24	7.4
Ferm 6	0.2	0.57	1.11	0.34	1.2	1.2	0.42	0.9	0.62	7.4
Ferm 7	0.4	1.14	2.22	0.68	0.6	1.2	0.21	0.9	1.24	3.7
Ferm 8	0.2	1.14	2.22	0.34	0.6	2.4	0.42	0.9	1.24	3.7
Ferm 9	0.2	0.57	2.22	0.34	1.2	1.2	0.21	1.8	1.24	3.7
Ferm 10	0.4	1.14	2.22	0.34	0.6	1.2	0.21	1.8	0.62	7.4
Ferm 11	0.4	0.57	1.11	0.34	0.6	2.4	0.21	1.8	0.62	3.7
Ferm 12	0.2	1.14	2.22	0.34	1.2	1.2	0.42	1.8	1.24	7.4
Ferm 13	0.4	1.14	2.22	0.68	1.2	1.2	0.21	0.9	0.62	7.4
Ferm 14	0.4	0.57	2.22	0.68	1.2	2.4	0.42	0.9	0.62	3.7
Ferm 15	0.4	1.14	1.11	0.68	0.6	2.4	0.42	1.8	1.24	7.4

Wort A.A.	**Met**	**Ile**	**Leu**	**His**	**Gly**	**Ala**	**Trp**	**Phe**	**Tyr**	**Pro**
Ferm 16	0.2	0.57	2.22	0.68	0.6	2.4	0.21	1.8	1.24	7.4
Ferm 17	0.4	1.14	1.11	0.34	1.2	2.4	0.21	1.8	0.62	7.4
Ferm 18	0.4	0.57	2.22	0.34	0.6	2.4	0.42	0.9	0.62	7.4
Ferm 19	0.2	1.14	2.22	0.68	1.2	2.4	0.21	0.9	0.62	3.7
Ferm 20	0.2	0.57	2.22	0.68	0.6	1.2	0.42	1.8	0.62	7.4
Ferm 21	0.2	0.57	1.11	0.68	0.6	2.4	0.21	0.9	1.24	7.4
Ferm 22	0.3	0.855	1.665	0.51	0.9	1.8	0.315	1.35	0.93	5.55
Ferm 23	0.2	1.14	1.11	0.34	1.2	2.4	0.21	0.9	1.24	7.4
Ferm 24	0.4	1.14	1.11	0.34	0.6	1.2	0.42	0.9	1.24	3.7
Ferm 25	0.2	0.57	1.11	0.34	0.6	1.2	0.21	0.9	0.62	3.7

2.6 Mashing trials

2.6.1 Mashing at 65°C

Mashing of 28 different malt types provided by Bairds Malt Ltd**,** (Witham, England) was carried out using the method described by Buckee *et al*. (1978). Sixty grams of each malt sample were ground (0.2mm, fine grind) into a beaker by using an automatic mill (BULHER Ltd, Leamington Spa, UK) and the contents mixed thoroughly. Fifty grams of ground material were transferred into a previously tared stainless steel mashing beaker, which was placed into a preheated CM-4 water mashing bath (Canongate Technology, Edinburgh, UK) at 65°C for 15min. From the remaining contents of the hopper, 5g were collected for determination of the moisture content and the hot water extract (HWE). Before the milling of each malt sample, the mill was rinsed with 20g of the malt sample to be ground. Distilled water (360ml) was added to each beaker containing the grist, at a temperature not exceeding 68°C and not below 66°C, to ensure an initial mash mix temperature of 65°C. When, all the beakers were filled with distilled water, the mechanical stirring devices were started. Lumps in the mash, were rapidly eliminated by stirring with a glass rod, which was rinsed between samples with a little distilled water at 65°C. Every beaker was covered with a 100mm watch glass to prevent losses due to evaporation and the mash was held at 65°C for 1h, after the hot water had been added to all the samples. After 1h, the mash was cooled to 15-25°C over a period not exceeding 10min. After cooling, the weight of each mash was adjusted to 450g, using distilled water at room temperature. The mashes were mixed thoroughly by stirring with a glass rod. Then, not before 30min and not after 45min from the beginning of the cooling step, the entire mash was decanted into a 32cm diameter fluted filter paper held by a funnel attached to a 500ml amber glass bottle. The first 50-100ml of the filtrate were returned to the filter and the filtrate continued to be collected. The filtration of the mashes was complete when the filter cake appeared dry (approximately after 2h). Twenty ml of each filtrate were used for the determination of the specific gravity at room temperature.

Five grams of grist, which were collected at the beginning of the milling, were transferred into a pre-weighed two-inch flat-bottom metal dish with a tight-fitting lid. The dish with the grist content was weighed and it was placed into an oven at 100°C for exactly 3h. After the 3 hour incubation, the lid was replaced and the dish was cooled in a

desiccator for 20min. Finally, the dish was re-weighed to calculate the loss of moisture. The moisture content of the sample was calculated with the following equation (8):

$$\textbf{Moisture Content} = \frac{W1 - W2 \times 100}{W2} \quad (8)$$

Where W1= Weight of sample before drying
W2= Weight of sample after drying

In order to calculate the excess degrees of gravity (**G**) of the filtrate the following formula (9) was used:

$$\textbf{G} \text{ (degrees Sacch)} = 1000 (SG-1) \quad (9)$$

Where SG = Specific gravity of the filtrate at $20^{o}C$

The hot water extract (**HWE**) of the 450g mash was calculated using the formula (10):

$$\textbf{HWE} \text{ (as is)} = \frac{G \times 8.773}{SG} \quad L^{o}/kg \quad (10)$$

Finally, the extract on dry basis was calculated with this formula (11):

$$\textbf{HWE} \text{ (dry)} = \frac{\text{HWE (as is)} \times 100}{100 - M} \quad (11)$$

Where M= Moisture Content (% m/m) of malt sample

2.6.2 Mashing at 4°C

The same procedure described above was used for this series of mashing experiments, with the only difference that mashing instead of taking place in a preheated CM-4 water mashing bath (Canongate Technology, Edinburgh, UK) at 65°C for 60min, it was carried out in an orbital shaking incubator (SANYO Gallenkamp, Watford Herts, UK) at speed of 250g at 4°C for 60min. In addition, the water that was used for the mashes, prior to the mashing step, had already been stored at 4°C for 24h.

These additional mashing experiments were conducted in order to investigate the actual percentage of free amino nitrogen produced during malting by the breakdown of the barley endosperm proteins and the percentage formed during mashing after the proteolysis of the mash proteins by the mash exopeptidases. Thus, by performing the mashing at 4°C, the mash proteases remained inactive under low temperature conditions and very low proteolytic activity was induced.

Chapter 3: Results

Continuously stirred fermentations were conducted in shake flasks on a rotary incubator using three different wort types (12°Plato and 20°Plato all malt worts and 20°Plato + 30% glucose adjunct wort), pitched with the same ale yeast strain (**No 70**). The spectrum of fermentation parameters determined included: yeast cell number, biomass, specific gravity, free amino nitrogen, pH, cell viability and fermentable sugar utilization were recorded and plotted at specific sampling times. Inadequate amino acid and ammonia utilization was observed for all the series of aerobic fermentations performed. Proline exhibited nearly no uptake. In addition, by examining the oligopeptide utilization for the normal gravity (12°Plato) all malt wort shake flask fermentations, it was observed that under continuous agitation conditions, yeast cells may be stressed and consequentially they may excrete/ secrete various proteinases, such as proteinase A, to carry out the catabolism of large molecular weight nitrogenous molecules, rather than taking up the individual wort available amino acids. Nevertheless, possible cell death and lysis during fermentations may have led to the release of intracellular yeast proteolylic enzymes into the fermentation environment. These released enzymes could have induced the degradation of large wort peptides and proteins into smaller peptides, which may have been utilized as additional nitrogen sources from the other viable cells. Total nitrogen mass balance calculations were carried out for all the fermentations in order to have a more improved idea about the unfermented and final fermented wort nitrogen distribution. Calculation of total free amino nitrogen, ammonia and amino utilization rates were also carried out.

Static fermentations were conducted in 2L cylinders. Two lager and two ale industrial production yeast stains were used, provided by Scottish and Newcastle. A similar wort type was used for all the fermentations (15°Plato malt + 30% VHM syrup). The two lager strains **SC3** and **SC4** and the ale strain **SC8** carried out the fermentations efficiently. The ale strain **SC5** exhibited a sluggish fermentation probably due to its sedimentation characteristics. Quantitative and qualitative identification and determination of wort nitrogen constituents (amino acids, oligopeptides and ammonium ions) was carried out. Adequate amino acid and ammonia absorption was observed for all fermentations. Little proline uptake occurred in all fermentations. The same effects were also observed when repitching. The amino acid utilization classification of Jones and Pierce (1964) was confirmed with some exceptions (details later).

Wort supplementations were carried out with two and five times, the natural wort

concentration of lysine, methionine and arginine. Ammonia supplementations were also conducted with two times, the initial wort levels. The yeast strain selected for the pitching of the fermentations was the lager yeast strain SC3. The lysine-supplemented fermentations were considerably faster (completed in 48h) than the control fermentations (completed in 96h). Lysine is a 'key' wort amino acid able to stimulate enhanced yeast metabolic activity. Similar effects were recorded with fermentations supplemented with arginine (completed in 67h). Supplementation of wort with methionine had an inhibitory effect on yeast fermentation (completed in 103h). Similar results were obtained, when wort was supplemented with ammonia (212h to completion).

A series of 26 fermentations supplemented with different mixtures of amino acids and ammonia was conducted. Five of the twenty six fermentations were complete within 56h, while the controls finished in 96h. A perception of key "marker" nitrogen wort constituents responsible for stimulating a sufficient yeast fermentative activity was achieved. Furthermore, the possibility of any synergistic effect between wort free amino acids and ammonia concerning improved yeast fermentation efficiency was studied. Final analysis of the VDK's, higher alcohol and ester levels was conducted to identify any "key" amino acid and ammonia combinations that may trigger the production of specific desirable or undesirable flavour compounds.

Small peptides are believed to be involved in the yeast nitrogen metabolism. Oligopeptide concentrations decreased and then accumulated at the end of the static fermentations. The total extracellular protease activity was determined and it was found that have followed a similar increase-decrease pattern to the oligopeptide spectrum. The excretion/secretion of yeast proteolytic enzymes under stress conditions, such as high gravity wort, could have resulted in the hydrolysis of larger peptides into small peptides, making them utilizable nitrogenous materials for yeast. This indicates that yeast metabolic activity does not cease when free amino nitrogen is depleted. However, again it should be added that proteolytic enzymes may have not be excreted by the stressed yeast cells, but they might have been released due to cell death and consequential lysis. Oligopeptide existence and utilization in unfermented wort probably has a beneficial effect on yeast's nutritional requirements. Lager and ale yeast strains can simultaneously use amino acids and small peptides as sources of assimilable nitrogen.

Nitrogenous wort components are partly formed during malting and partly during mashing due to the action of barley proteases on hordein. The percentages reported in literature are: 70% of FAN derived during malting and 30% during mashing. Laboratory mashing was

performed at 65°C for 28 different malt types. An interesting variation of FAN levels was observed with the different varieties of malts. Laboratory mashing was also repeated at 4°C in order that inactivation of barley proteases is achieved. This was conducted in order to investigate if these values found in other publications are valid and realistic. From our results, it was found that approximately 90% of total FAN originates during malting and the remaining FAN during mashing.

3.1 Shake flask fermentations

The shake flasks fermentations (12°Plato and 20°Plato all malt worts and 20°Plato + 30% glucose adjunct wort) were conducted in order to investigate the yeast fermentation performance and nitrogen metabolism under conditions of continuous aeration achieved with constant wort agitation.

3.1.1 12°Plato wort

3.1.1.1 Fermentation profile and sugar utilization (12°Plato)

Figure 22 illustrates the free amino nitrogen (FAN), wort gravity and pH reduction and the yeast cell number, biomass and viability. In addition, the total utilization of sugars during the course of fermentation is presented in **Figure 22D**. The time taken to reach the target gravity (3°Plato) for this series of shake flask fermentations was 120h. The yeast strain selected to carry out these fermentations was the ale strain No 70. Maximum cell number was obtained during the third day of fermentation (15.6×10^7cells/ml), thereafter the death phase started, confirmed by viability measurements of the cells. FAN wort levels reduced steadily for 72h and then underwent a slight increase until the completion of fermentation. Cell viability remained high (96%) for the first 3 days of fermentation and then it declined sharply, dropping from 96 to 86% viable cells. Cell biomass reached its highest value after 72h of incubation, which was estimated to be 3.8mg/ml of sample. Thereafter, biomass levels declined with the course of time. Specific gravity (S.G.) followed the same rate of reduction as FAN. Both of these parameters simultaneously decreased for the first 72h of incubation and it can be said that these two values are proportional. S.G. then exhibited a plateau pattern until the end of the fermentation. The pH gradually declined for the first 72h of fermentation and then it started increasing again until the end of the fermentation. The

fourth plot (**Figure 22D**) depicts the utilization of wort carbohydrates during fermentation. Glucose and sucrose were completely metabolized by yeast cells during the first 24h incubation followed by fructose and maltose, whose utilization was completed after 48 and 72h incubation, respectively. Maltotriose was poorly utilized and no significant decrease from its initial level was observed.

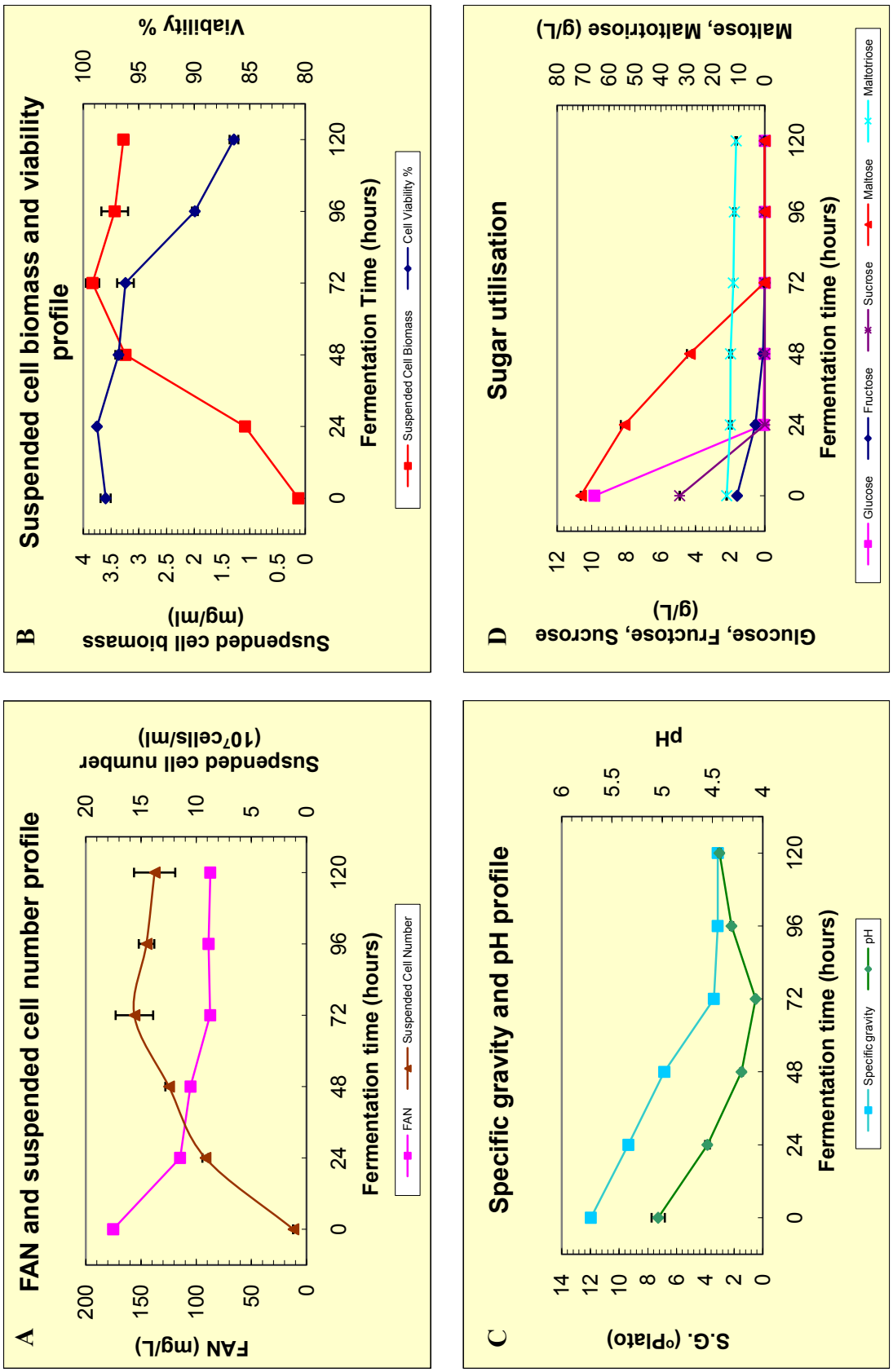


Figure 22: Fermentation profile and sugar utilization for the shake flask ale (No 70) fermentations (12°Plato). The results are the mean values of three fermentations ± S.D.

3.1.1.2 Amino acid and ammonia utilization (12°Plato)

Figure 23 illustrates the amino acid and ammonia consumption according to their absorption rates reported by Jones and Pierce (1964), for 12°Plato wort ale fermentations. It should be added that ammonia has been classified initially by Jones and Pierce (1964) as Group 3 assimilable nitrogen source but in these studies, ammonia absorption is plotted with the same system of axes as proline for practical reasons.

In more detail, the first graph (**Figure 23A**) shows the amino acids which should have exhibited rapid absorption. However, such an observation is not valid due to the fact that only arginine and lysine appeared to have followed this absorption pattern, by being totally removed from the wort during the first 48h of fermentation. Glutamic acid concentration, instead of being entirely absorbed during fermentation, increased after the first day of incubation. The remaining amino acids, which belong in the same group, were not consumed until the end of fermentation.

The second graph (**Figure 23B**) illustrates the concentration of amino acids believed to be utilized intermediately. As it can be seen again, none of the amino acids present in wort, except methionine, were totally used by the yeast cells. Methionine could not be detected after the second day of fermentation.

The third graph (**Figure 23C**) shows the amino acid uptake during the course of fermentation for amino acids being classified in the third group. All the amino acids exhibited no or very negligible absorption.

The last plot (**Figure 23D**) illustrates the utilization of proline and ammonia during fermentation. As was expected, proline remained unused even after 120h of fermentation. It seems that the ammonia concentration was gradually reduced until the third day of fermentation and thereafter its levels started to build up in the fermentation medium until the target gravity of fermented wort was achieved.

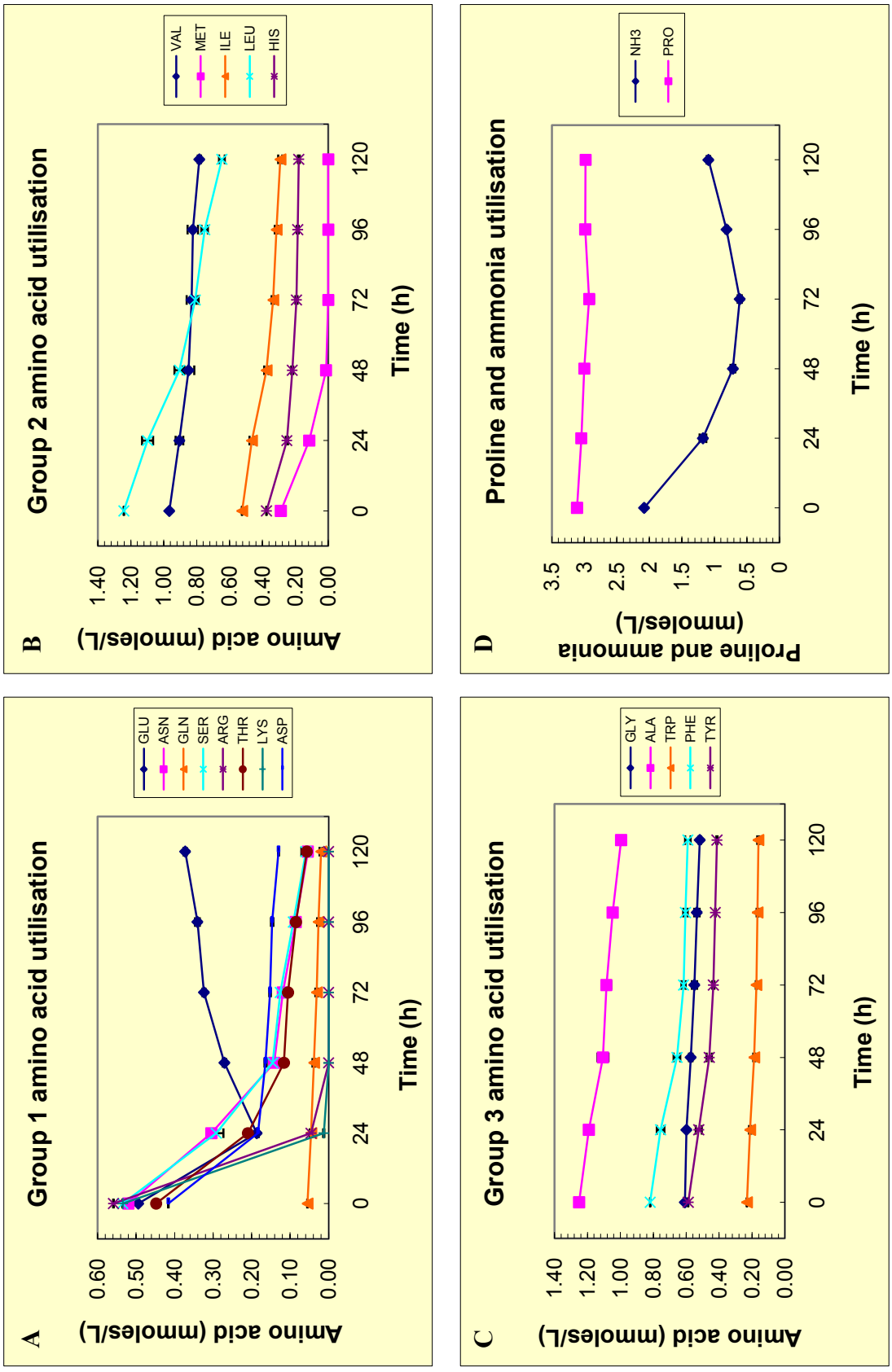


Figure 23: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the 12°Plato ale strain No70 shake flask fermentations (the results are the mean values of three fermentations ± SD.)

3.1.1.3 Wort nitrogen distribution and nitrogen source utilization (12°Plato)

Figure 24 illustrates the detailed nitrogen distribution before and after fermentation in terms of the percentage of total nitrogen, each individual amino acid and ammonia constitutes for the shake flask fermentations conducted with a 12°Plato all malt wort. In addition, **Figure 24C** shows the relative nitrogen utilization for every single wort nitrogenous material.

As it can be seen, the amino acids that compose the smallest percent in unfermented wort are methionine and tryptophan with only 0.2% and 0.1% of total wort nitrogen content, respectively. On the other hand, amino acids with the highest contribution of assimilable nitrogen are alanine (1.6%), leucine (1.1%) and valine (0.9%). The most abundant of the wort nitrogenous materials were found to be ammonia, which constitutes almost the 14% of the total yeast assimilable nitrogen and proline, which composes the 3% of free amino nitrogen. All the spectrum of wort amino acids including ammonium ions comprises 26% of total unfermented wort nitrogen content. The remaining 74% of the total wort nitrogen is also part of yeast's assimilable nitrogen and is believed to be small peptides with two to three amino acid residues.

As can be observed from the last plot of this figure (**Figure 24C**), during these shake flask ale fermentations, the only nitrogen compounds that have undergone complete utilization were the Group 1 amino acids, arginine and lysine and the Group 2 amino acid, methionine. Surprisingly, only 25% of glutamic acid's initial concentration in unfermented wort was utilized during these fermentations, which is a paradox since glutamic acid is usually the first amino acid that is completely utilized within the first 19-24h of fermentation. In addition, glutamic acid, which according to the literature is rapidly exhausted during the first hours of fermentation (Jones and Pierce, 1964), not only exhibited a very poor utilization rate, but it also started to be released in the fermenting wort after 24h of yeast incubation. A similar observation was also valid for Group 3 amino acids, whereas only the 24% of their initial levels was found to have been consumed. Less than half of the original ammonia wort concentration was found to have been used by the yeast at the end of the fermentation.

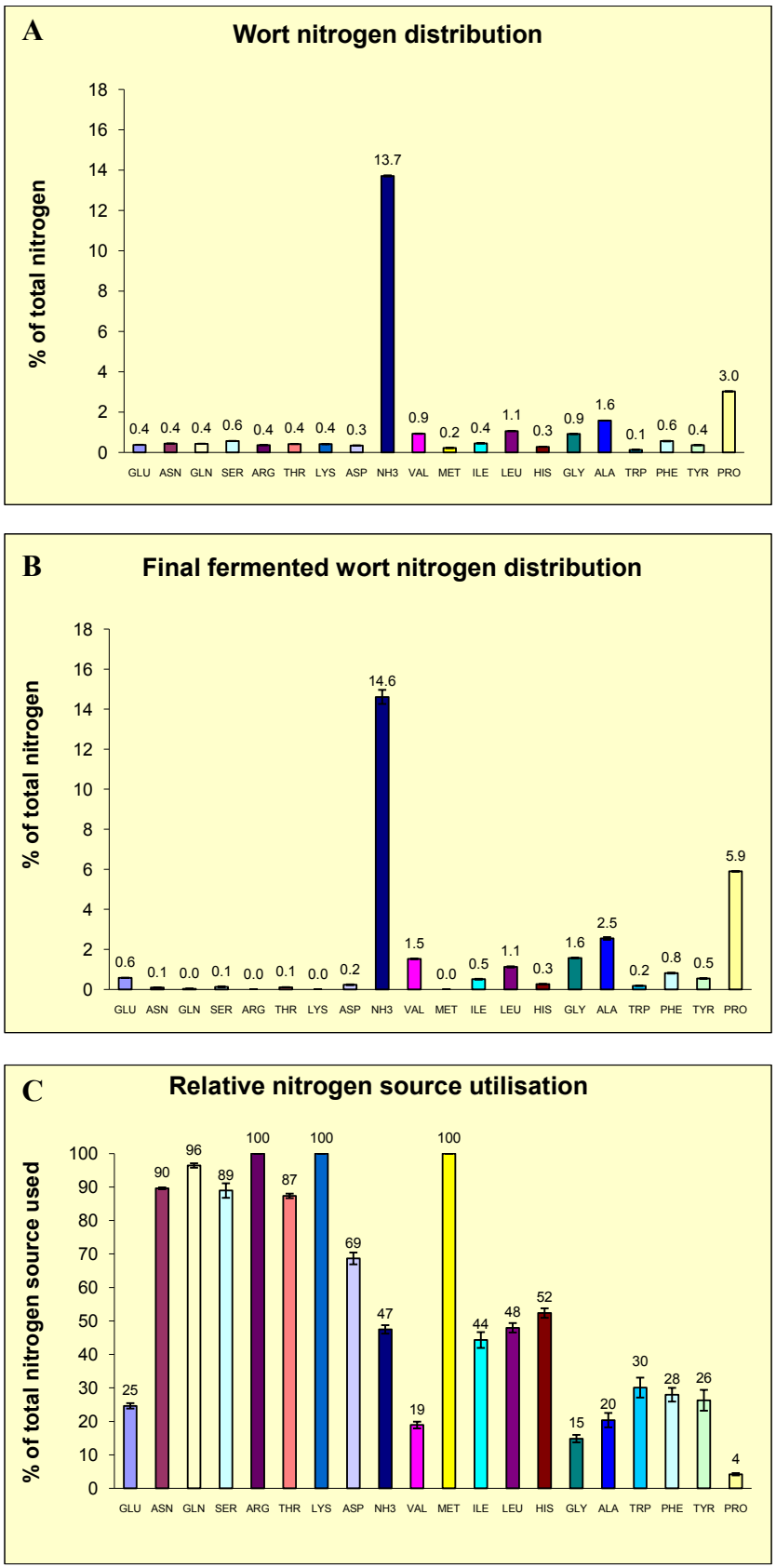


Figure 24: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (12^{o}Plato shake flasks). The results are the mean values of three fermentations ± S.D.

3.1.2 20^{o}Plato wort

3.1.2.1 Fermentation profile and sugar utilization (20^{o}Plato)

Figure 25 illustrates the fermentation profile for FAN, S.G., pH, cell number, biomass, viability and sugar uptake. The incubation time needed for these high gravity wort (20^{o}Plato) shake flask fermentations to reach the target gravity (3^{o}Plato) was 120h. The same ale yeast strain (No 70) was also used for these series of experiments as it was for the 12^{o}Plato shake flask fermentations.

Maximum cell number was achieved during the third day of the experiment and it was estimated to be 25x10^{7}cells/ml. Thereafter, the concentration of cells in suspension started to decline until the end of the fermentation. The concentration of wort assimilable nitrogen appeared to decrease progressively for the first 72h of fermentation and subsequently the FAN wort content started to slightly increase until the fermentation ceased.

Maximum cell biomass peaked at 6.6mg/ml of sample 72h into fermentation and then it declined. Cell viability did not exhibit a significant decrease, having decreased only by 4% from the yeast pitching level. Specific gravity decreased gradually for the first 72h of fermentation and then its value remained more or less constant until the end of the experiment.

As with FAN, pH also reduced in harmony from 5.04 to 4.15, for the first 3 days of fermentation and then started increasing again to 4.35 and then it reduced again back to 3.99, until the fermentation ceased.

The fourth plot (**Figure 25D**) depicts the utilization of wort sugars during fermentation. As it can be observed although glucose wort levels were very high (32g/L), this sugar was consumed within the first 24h of fermentation. Sucrose was also totally utilized at the same time as glucose, followed by fructose and maltose, which were depleted after 48 and 96h, respectively. Finally, maltotriose exhibited a very poor utilization throughout the course of the fermentation.

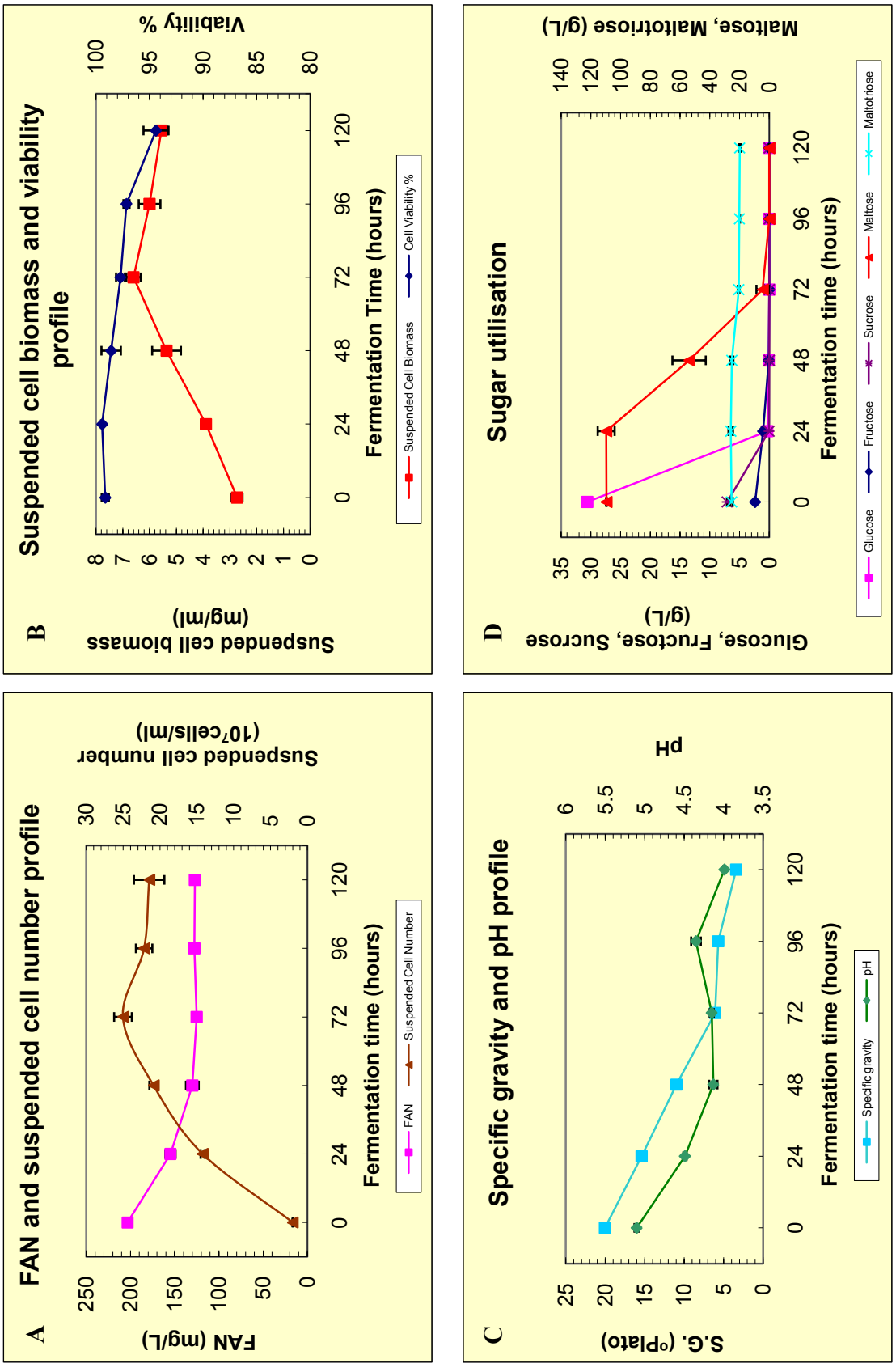


Figure 25: Fermentation profile and sugar utilization for the shake flask No 70 ale fermentations (20°Plato). The results are the mean values of three fermentations ± S.D.

3.1.2.2. Amino acid and ammonia utilization (20°Plato)

The amino acid and ammonia uptake is presented in **Figure 26** for the 20°Plato wort fermentations, which were conducted in shake flasks and pitched with the ale yeast strain No 70.

In more detail, amino acids classified in the first group were not taken up entirely or very rapidly by the yeast cells. As was also observed during the 12°Plato shake flask fermentations, only arginine and lysine were completely utilized after the second day of fermentation. Glutamate again repeated the same absorption behaviour as with the 12°Plato wort experiments, whose concentration began to increase after the first 24h of incubation. Aspartic acid was used during the first 24h and then its concentration remained almost unchanged for the rest of the fermentation. A similar effect was also observed for the amino acids asparagine, threonine and serine, which were utilized steadily during the first 48h and after that their levels plateaued. Surprisingly, glutamine, which is considered as a very good nitrogen source for yeast (Jones and Pierce, 1964), is always taken up during the early stages of fermentation, was not preferred by yeast cells this time and its initial wort concentration remained intact until the end of the experiment.

Amino acids, which have been categorized in the second group, exhibited a very poor utilization. All of them, apart from methionine, were found in high residual amounts in the fermented wort. Methionine was fully consumed 72h from the start of fermentation.

Practically no absorption of the amino acids of the third group was detected during fermentation and the wort concentrations of these particular amino acids continued to be constant during the course of yeast growth.

As would be expected, proline was not used by the yeast while, ammonia levels decreased until the last day of the experiment, but without being totally consumed.

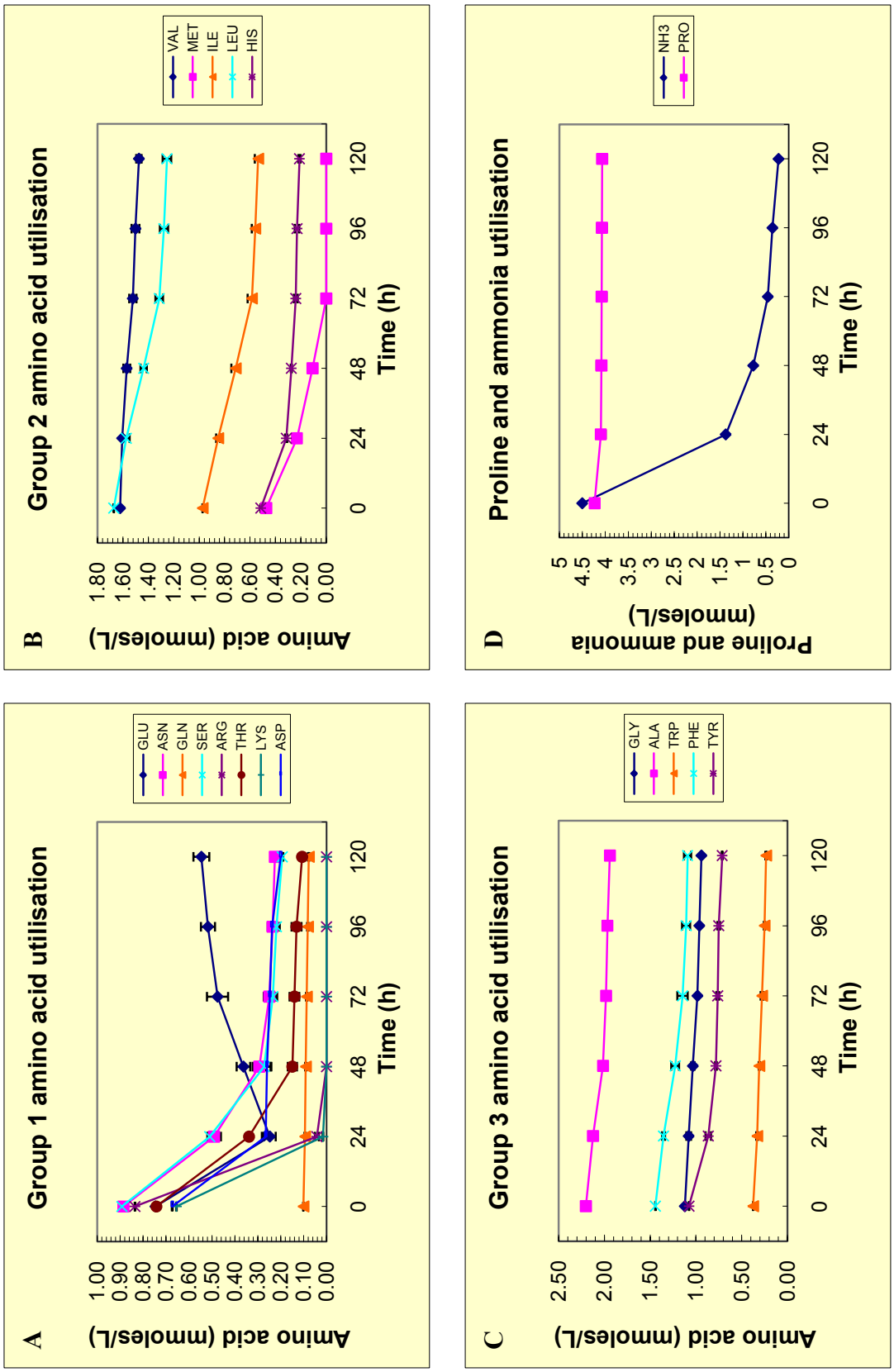


Figure 26: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the No 70 ale shake flask fermentations (20°Plato). The results are the mean values of three fermentations ± S.D.

3.1.2.3 Wort nitrogen distribution and nitrogen source utilization (20°Plato)

The detailed nitrogen distribution, before and after fermentation, is illustrated in **Figure 27**. What percentage of total nitrogen, each individual wort amino acid and ammonia constitutes before and after fermentation for the 20°Plato all malt wort shake flask fermentations, is shown in **Figures 27A** and **27B**. In addition, the last plot of this figure (**Figure 27C**) shows the relative nitrogen utilization for every single wort assimilable nitrogenous material.

As it can be seen, the amino acids that belong in to the Group 1 constitute only 4% of the total nitrogen in unfermented wort. On the other hand, amino acids of the group 2 comprise 3.8% of the yeast's total assimilable nitrogen. Group 3 amino acids constitute 5.4% of the total wort nitrogen concentration. The most abundant of the wort nitrogenous materials were found to be ammonia, which represents almost the 26% of the total yeast assimilable nitrogen and proline, which composes 3.6% of free amino nitrogen. The sum of wort amino acids and ammonia comprises 41.5% of total unfermented wort nitrogen content and the remaining 58.5% is believed to be mainly oligopeptides composed of two to three amino acids.

As it can be observed from the last plot of this figure (**Figure 27C**), during this series of shake flask ale fermentations, the only nitrogen compounds that have depleted, were the amino acids, arginine, methionine and lysine, as occurred during the normal gravity wort shake flask fermentations (12°Plato). Surprisingly, 10-15% more tryptophan and tyrosine were used than glutamine, which is a nitrogen source that exhibited complete absorption during the early stages of fermentation. A similar observation was also valid for glutamate compared to these two Group 3 amino acids, but that was mainly an effect of glutamate increase in the fermentation medium, after 24h of fermentation. This time, almost the entire concentration of the original wort ammonia (95%) was found to have been used by the yeast by the end of the experiment.

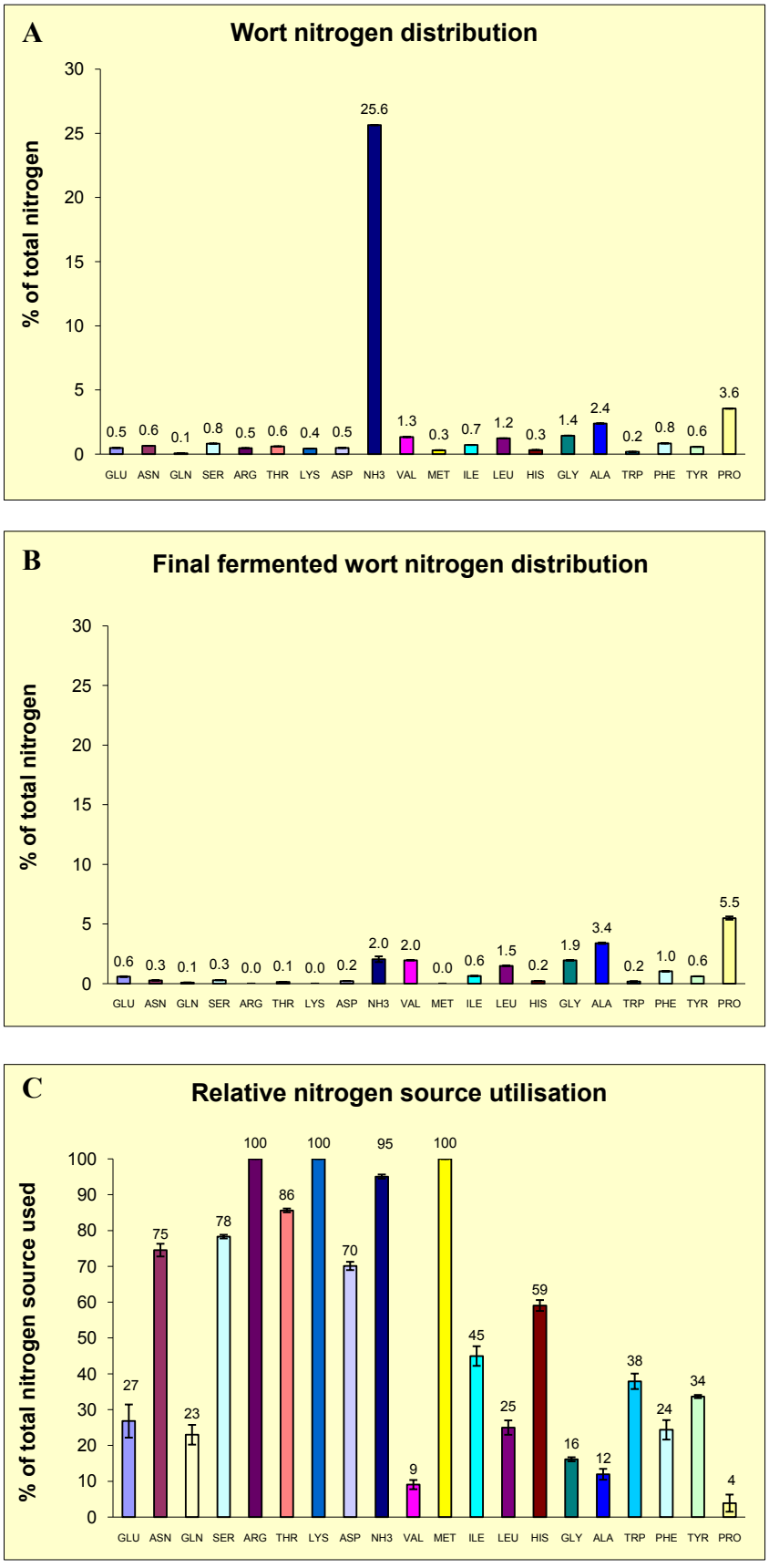


Figure 27: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (20°Plato shake flasks). The results are the mean values of three fermentations ± S.D.

3.1.3 20°Plato wort + 30% Glucose

3.1.3.1 Fermentation profile and sugar utilization

Figure 28 depicts an outline of the parameters measured during the shake flask fermentations, in which a high gravity adjunct wort type was used (20°Plato + 30% Glucose). This series of experiments lasted longer than the other stirred fermentations and the target gravity (3°Plato) was obtained after 144h. Once again, the same ale yeast strain was used during these fermentations.

Yeast cells multiplied gradually reaching their maximum level after 72h of fermentation ($17.7x10^7$cells/ml). Thereafter, the number of dividing cells reduced steadily until the experiment ceased. The total assimilable nitrogen concentration started decreasing from the beginning of fermentation until 120h incubation and then it appeared to have undergone a slight increase for the last 24h of fermentation.

Moving on the second graph of this figure (**Figure 28B**), in which cell biomass and viability is depicted, it can be said that the yeast cells remained viable for the first 48h of fermentation and then their viability decreased gradually below 90% at the end of the experiment. In addition, maximum cell biomass peaked at 5.4mg/ml of sample 72h from the start of fermentation.

Specific gravity decrease followed a linear reduction pattern during the course of fermentation having reached its target value after 6 days incubation. The pH dropped from 5.2 to 4.2 within 72h of fermentation and then as occurred with FAN, it underwent a slight increase.

With sugar uptake, sucrose was the first wort carbohydrate to have undergone complete consumption within 24h incubation. Glucose and fructose were depleted after 72h of incubation and maltose was fully absorbed during the 6th day of the experiment. Maltotriose appeared not to have been utilized at all throughout the fermentation.

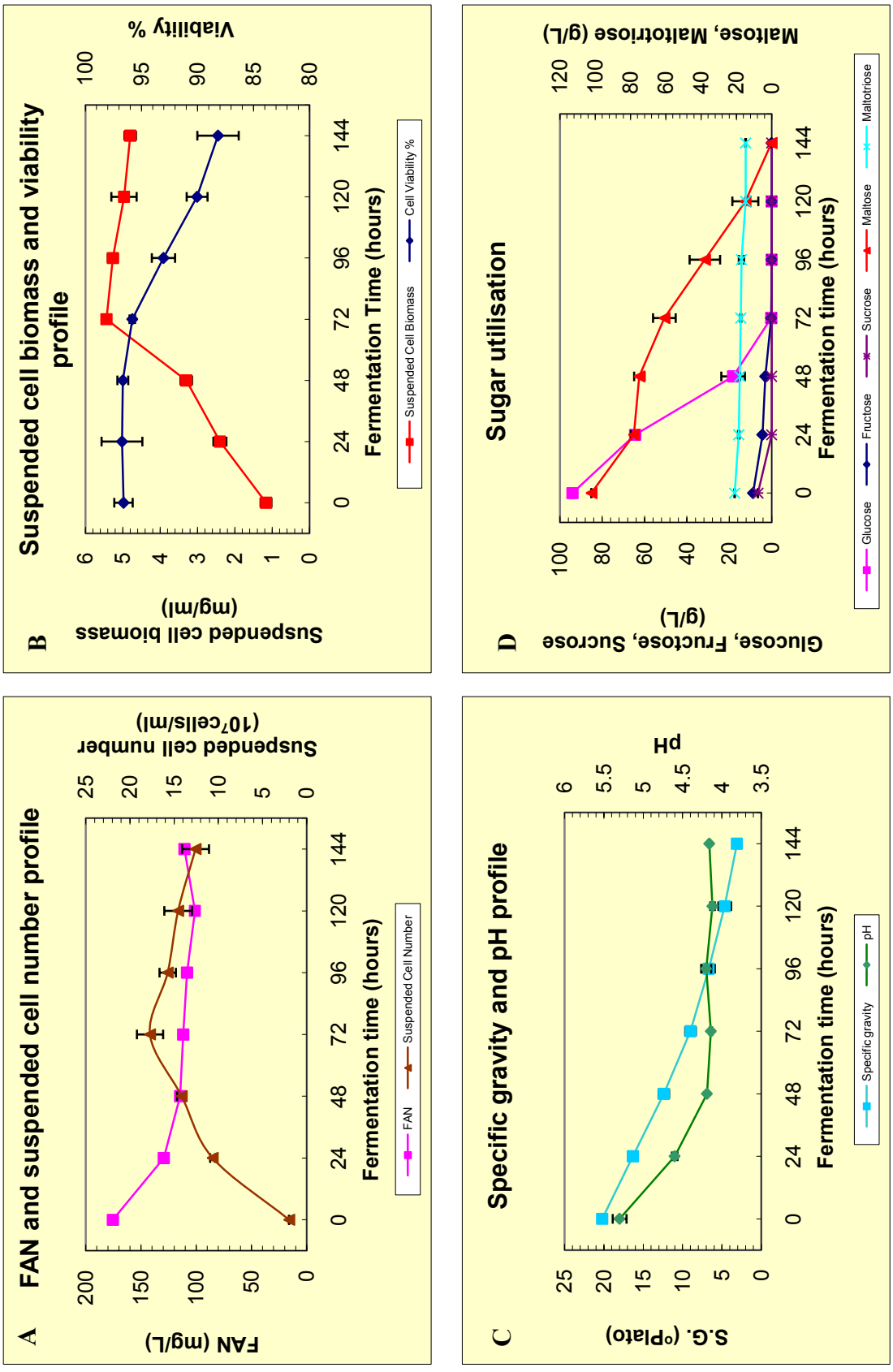


Figure 28: Fermentation profile and sugar utilization for the shake flask No 70 ale fermentations (20°Plato + 30% Glucose). The results are the mean values of three fermentations ± S.D.

3.1.3.2 Amino acid and ammonia utilization (20°Plato + 30% Glucose)

The general amino acid and ammonia consumption during the shake flask fermentations, which were carried out with a high gravity adjunct wort (20°P + 30% Glucose), are shown in **Figure 29**.

Again, complete utilization of Group 1 amino acids only occurred for arginine and lysine after 2 days fermentation. Glutamic acid was also detected to have been excreted in the fermentation broth after the first day of fermentation, since its concentration continuously increased during fermentation. The majority of the remaining amino acids in the first group were utilized steadily during fermentation, but none of them was completely removed from the fermenting wort. In addition, the uptake of glutamine seemed to have followed a similar pattern to proline, whereas no utilization of this particular amino acid appeared to have taken place.

In the second graph (**Figure 29B**), where Group 2 amino acid utilization rates are plotted, once again it was found that methionine was the lone free amino nitrogen source that was consumed entirely. All the other amino acids in this group and the amino acids in the third group did not play any nutritional role in the yeast's nitrogen metabolism, since they did not exhibit any uptake.

The single amino acid of the fourth group, proline, was not utilized at all by the yeast. Wort ammonia levels started to decrease progressively from the beginning of the experiment until the end of fermentation with trace levels of ammonia found in the fermented wort.

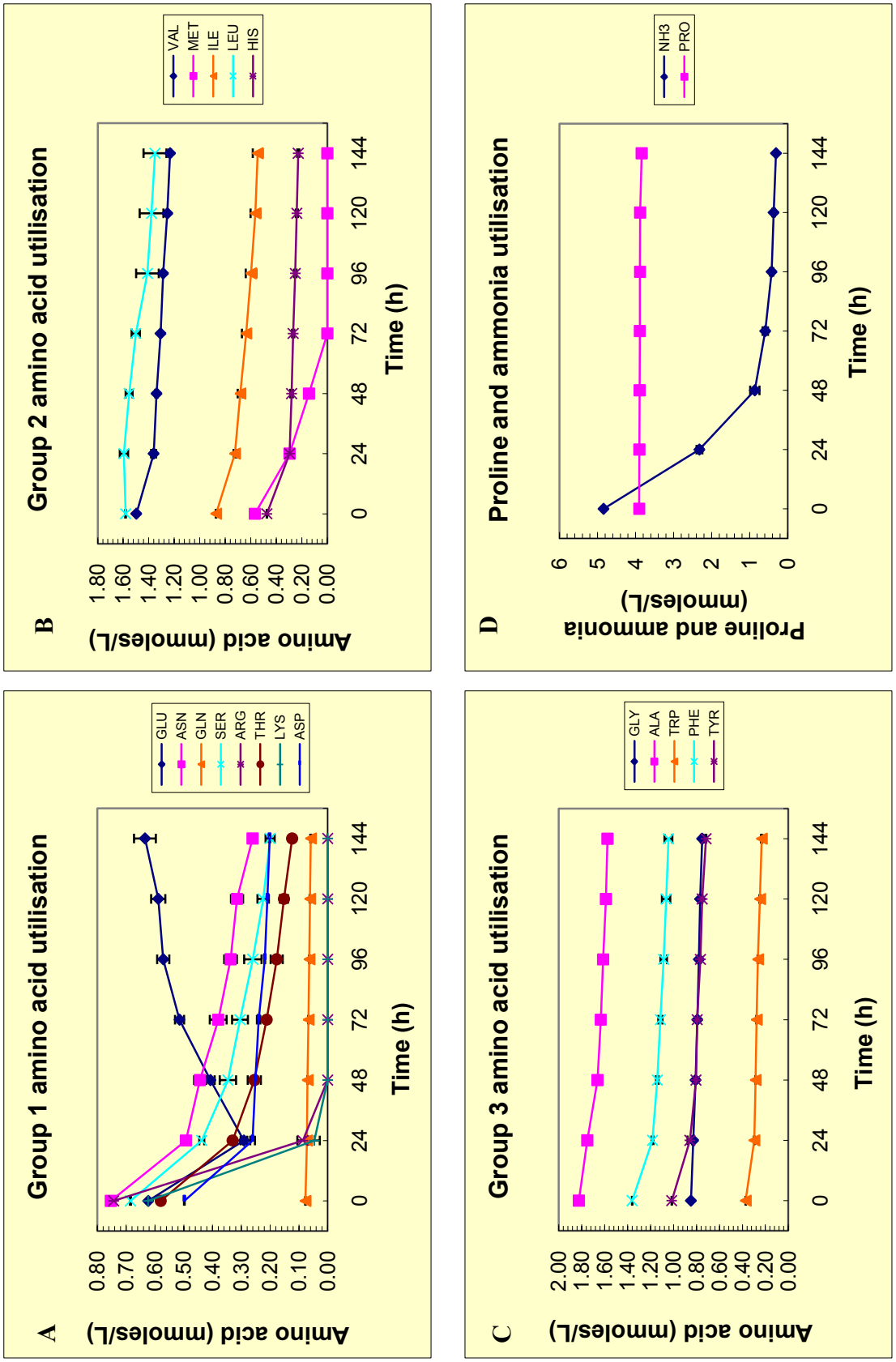


Figure 29: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the No 70 ale shake flask fermentations (20°Plato +30% glucose). The results are the mean values of three fermentations ± S.D.

3.1.3.3 Wort nitrogen distribution and nitrogen source utilization (20°Plato + 30% Glucose)

Figure 30 illustrates the detailed nitrogen distribution before and after fermentation in terms of the percentage of total nitrogen each individual amino acid and ammonia constitutes for high gravity shake flask fermentations conducted with a 20°Plato wort containing 30% glucose adjunct. The last plot of this figure (**Figure 30C**) shows the relative nitrogen utilization for each single wort nitrogenous material.

Amino acids that belong in to Group 1 constitute only 3.8% of the total nitrogen content in unfermented wort. Again, amino acids of Group 2 comprise 4.1% of the total yeast assimilable nitrogen. Group 3 amino acids constitute 5.3% of the total wort nitrogen concentration. It is well documented that the most abundant wort amino acid is proline (Jones and Pierce, 1964; Taylor, 1983; Jin *et al*. 1996), which comprises nearly the same percentage as Group 3 amino nitrogenous nutrients. Ammonia, this time appeared to represent almost 32% of the total wort utilizable nitrogen. Wort amino acids and ammonia comprise 48% of the total unfermented wort nitrogen content and the remaining 52% is believed to be single oligopeptides that also play a significant role in yeast's nitrogen metabolism (Dalme and Thorne, 1949; Macwilliam and Clapperton, 1969; Clapperton, 1971a; Clapperton, 1971b; Marder *et al.* 1977; Nisbet and Payne, 1979; Ingledew and Patterson, 1999; Patterson and Ingledew, 1999).

Again, the only nitrogen compounds that have undergone complete utilization were the amino acids arginine, methionine and lysine, as occurred during the previous normal and high gravity shake flask fermentations, where the same ale yeast strain was used (No 70). The negative percentage of glutamic acid absorption (-2%) explains the fact that was also shown in the amino acid utilization section, where glutamate commenced to be released into the fermentation environment after 24h of fermentation and at the end of the fermentation period, 2% more than in the unfermented wort was detected in the final fermented wort.

Similarly with the 20°Plato all malt wort fermentations, 10-15% more tryptophan and 5% more tyrosine were used than glutamine. Again only 6% of the original wort ammonia concentration remained unmetabolised, suggesting that ammonia is preferred as a nitrogen source during high gravity fermentations compared to the normal gravity wort fermentations.

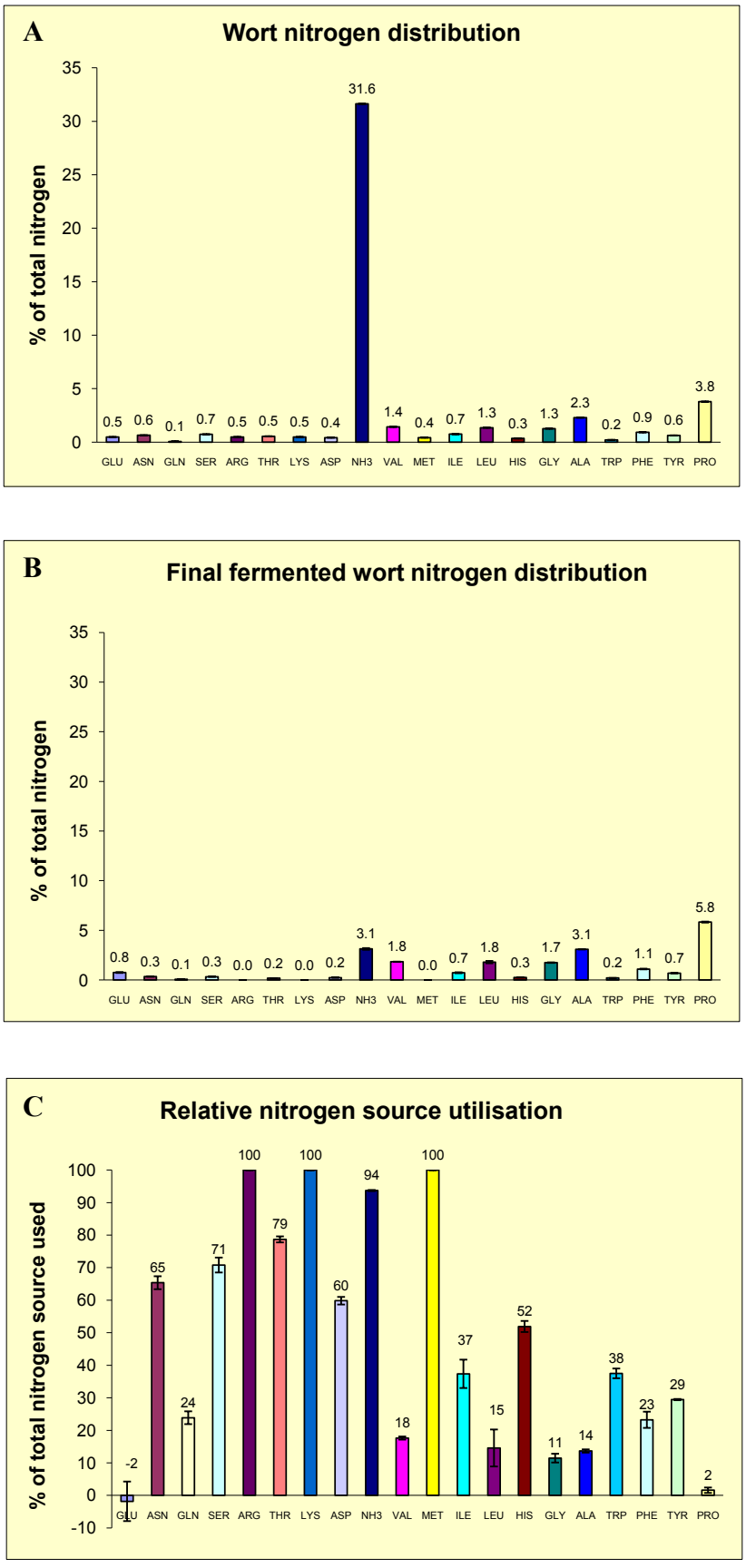


Figure 30: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (20°Plato +30% Glu shake flasks). The results are the mean values of three fermentations ± S.D.

3.2 Static fermentations

3.2.1 Lager fermentations

As described in the Materials and Methods section, two industrial lager yeast strains, **SC3** and **SC4**, supplied by Scottish Courage, were used for conducting this series of static anaerobic fermentations being carried out in 2L cylinders.

3.2.1.1 SC3 lager fermentations

3.2.1.1.1 Fermentation profile and sugar utilization

Figure 31 illustrates the fermentation profile for the static lager fermentations pitched with yeast strain SC3. The incubation time needed to reach the target gravity (3°Plato) was 96h. Maximum cell concentration was obtained during the first day of fermentation, which peaked at $10x10^7$cells/ml. At the next sampling point (43h), it was observed that the suspended cell number had decreased to less than half of its maximum value. FAN decreased in steps for the first 48h of the experiment and then appeared to increase slightly until the end of the fermentation. Maximum cell biomass was achieved during the first 24h of fermentation (3.1mg/ml of sample), and then it declined gradually until the end of the incubation. Cell viability did not undergo a significant decrease having reduced only by 1% from the inoculation of the yeast slurry.

Specific gravity decreased gradually throughout the experiment achieving its target level (3°Plato) after 4 days fermentation. The pH reduced in harmony with FAN for the first 67h of fermentation and then it started increasing again until the fermentation stopped.

Complete utilization of wort carbohydrates glucose, sucrose and fructose was achieved after 96h fermentation (**Figure 31D**). Maltose reduced progressively during the incubation with a sharp decrease occurring between 0h and 43h. Nevertheless, yeast cells did not manage to metabolize all the wort sugar and significant residual levels of maltose were found in the fermented wort. Surprisingly, maltotriose seems to have been preferred to maltose by this yeast strain according to its uptake rate.

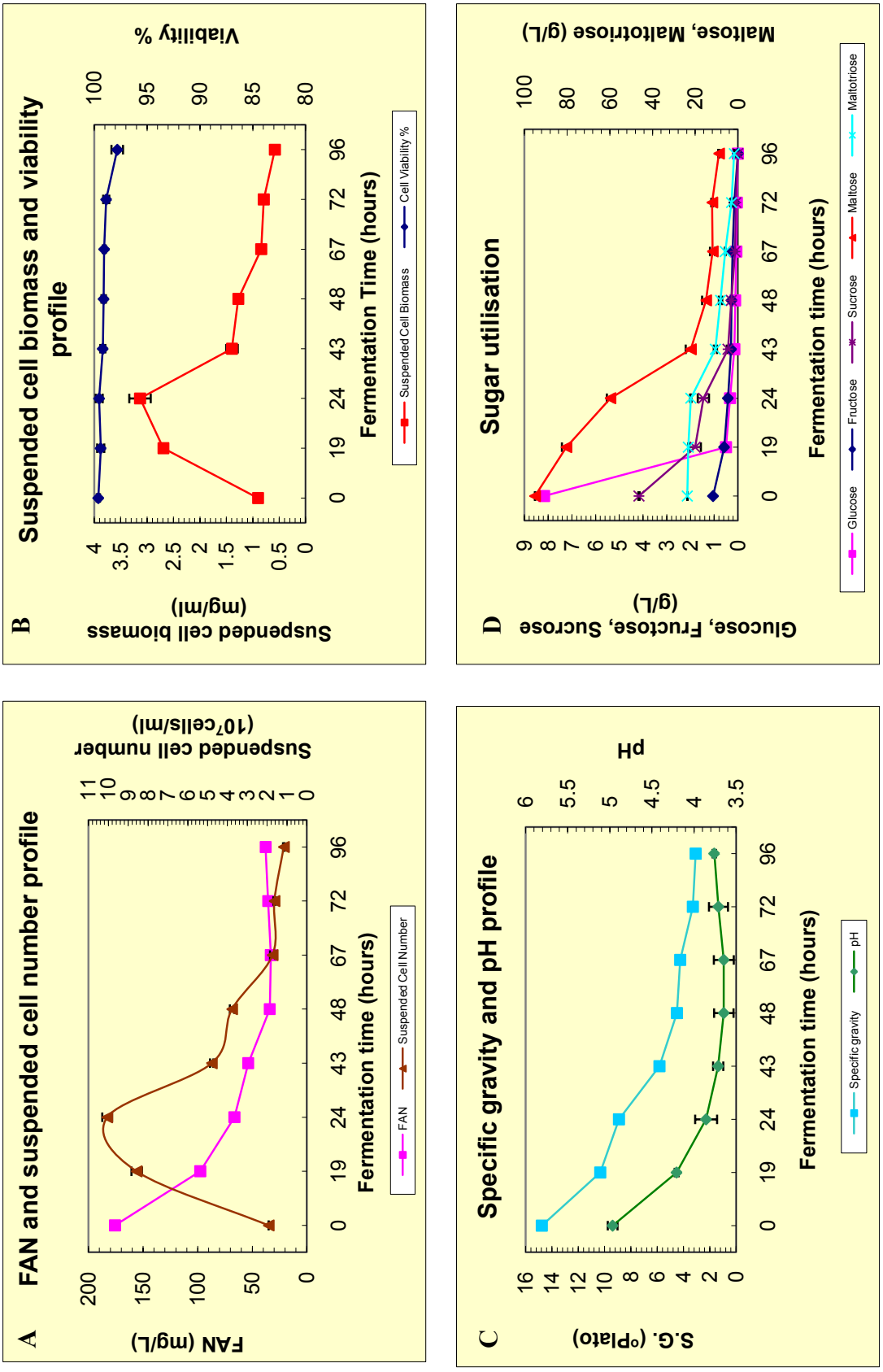


Figure 31: Fermentation profile and sugar utilization for the SC3 lager static fermentations. The results are the mean values of three fermentations ± S.D.

3.2.1.1.2 Amino acid and ammonia utilization

Figure 32 shows the nitrogen metabolism during static wort fermentations in which the lager yeast strain SC3 was used as the inoculum.

In **Figure 32A**, the sum of the amino acids of Group 1 was utilized rapidly within the first day of fermentation. Threonine and arginine were entirely taken up after 43h of fermentation. No residual amino acid levels found in the fermenting medium after 48h of fermentation. However, glutamine was the exception to this assimilation pattern because its concentration was depleted within the first 19h of fermentation.

A similar effect was also observed for methionine, which was taken up by 24h fermentation. In addition, isoleucine and histidine, which are also constituents of the second group, excluding valine, were depleted 43h into the fermentation. Valine and leucine concentrations were totally assimilated by the yeast during the second day of the experiment.

Similarly, Group 3 amino acids, phenylalanine, tryptophan and tyrosine were exhausted at the same time as the amino acids of the second group, valine and leucine. By examining the metabolic behaviour of the most abundant amino acid found in wort, no proline utilization occurred during these fermentations using the industrial yeast strain SC3. Proline concentration remained unchanged throughout the incubation period. Ammonia, on the other hand, was consumed steadily for the first 43h of fermentation and then its concentration remained more or less the same until the end of the fermentation.

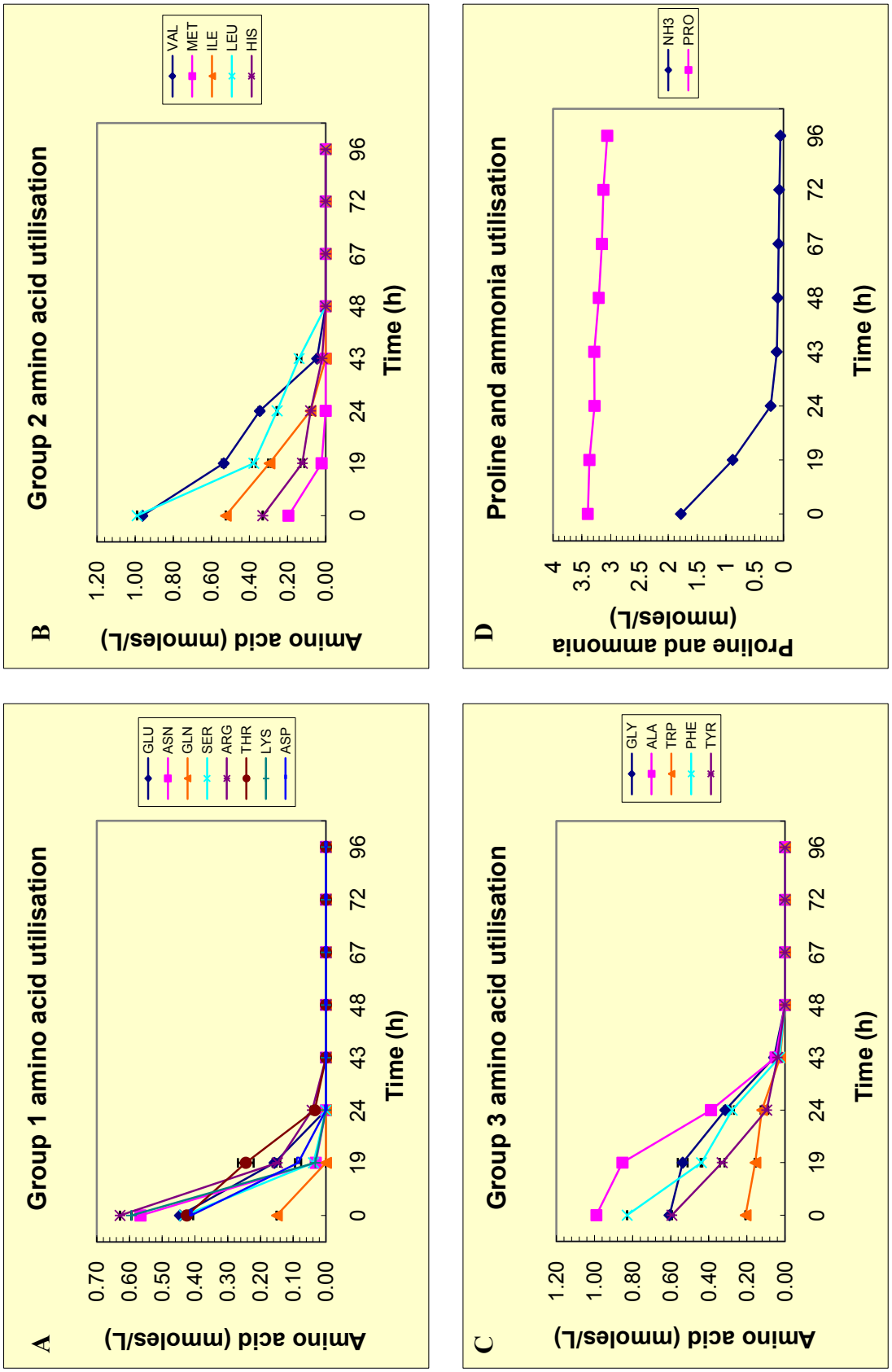


Figure 32: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the SC3 lager static fermentations. The results are the mean values of three fermentations ± S.D.

3.2.1.1.3 Wort nitrogen distribution and nitrogen source utilization

Figure 33 illustrates the detailed nitrogen distribution before and after fermentation in terms of percentage of total nitrogen each individual amino acid and ammonia constitutes. This pro and pre nitrogen orientation is for the static lager fermentations conducted with the yeast strain SC3. The last plot of this figure (**Figure 33C**) shows the relative nitrogen utilization for every single wort nitrogenous material.

As it can be seen, the amino acids that compose the smallest percentage in unfermented wort are glutamine, methionine and tryptophan. On the other hand, amino acids with the highest percentages are valine, glycine and alanine. The most abundant of the wort nitrogenous materials were ammonia, which constitutes 11.5% of the total yeast assimilable nitrogen and proline, which composes 3.2% of free amino nitrogen.

As it can be observed from **Figure 33C**, during these static lager fermentations, all the amino acids were consumed efficiently apart from proline, (only 6% of its initial wort content was utilized) and 90% of the ammonia wort levels.

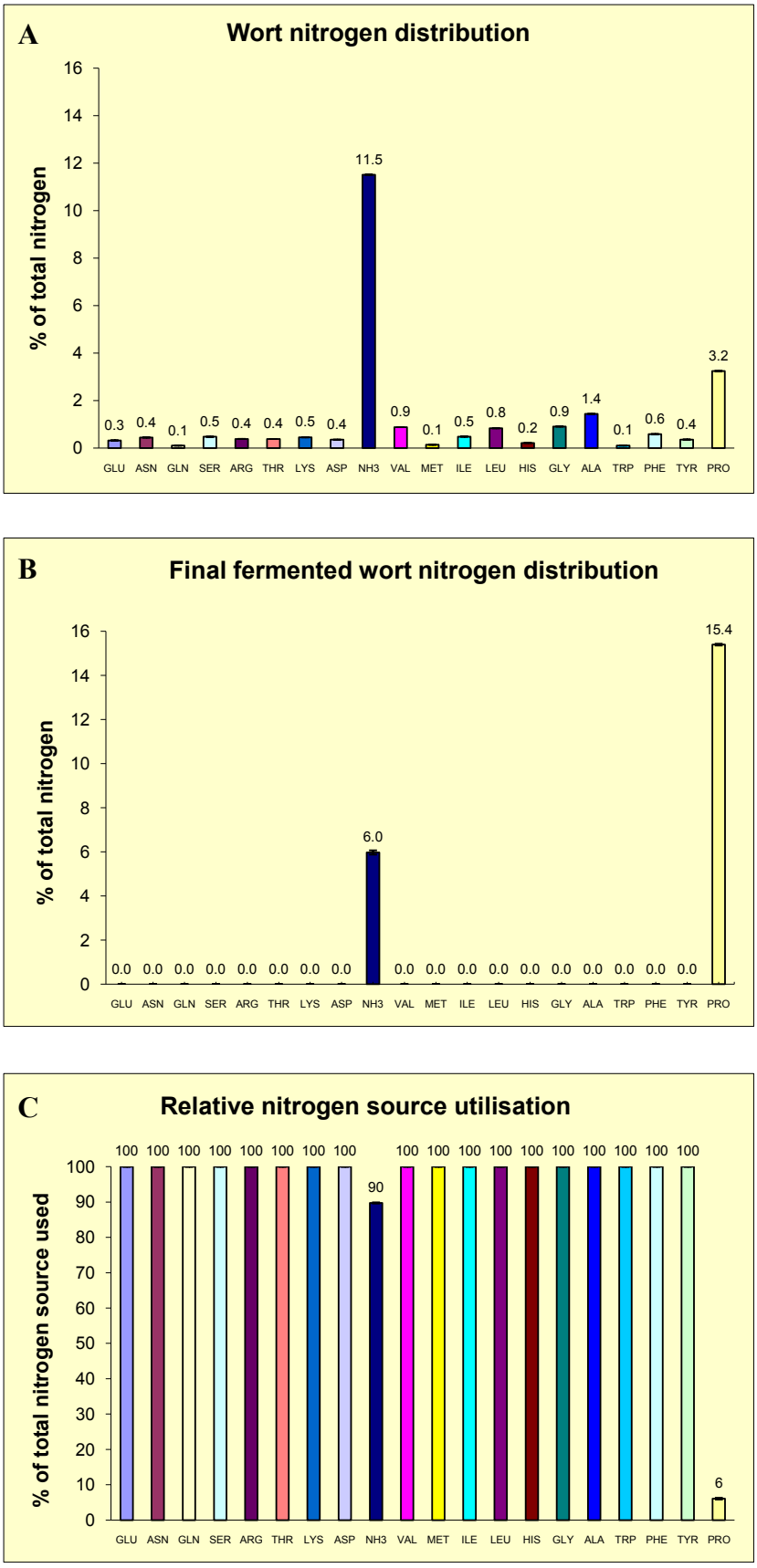


Figure 33: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (SC3 lager static fermentations). The results are the mean values of three fermentations ± S.D.

3.2.1.1.4 Final fermentation measurements

Table 8: Final measurements for **SC3 lager** fermentations
(after 96h fermentation at 15°C and incubation at 4°C for 24h)

Parameter / Cylinder	ABV (%)	Total Wet Yeast Biomass (g)	Total Dry Yeast Biomass (g)	Yeast Viability (%)
Cylinder 1	6.591	33.769	25.510	98.35
Cylinder 2	6.293	32.250	24.127	97.28
Cylinder 3	6.141	31.376	22.907	99.86
Average ± S.D.	**6.342 ± 0.229**	**32.465 ± 1.211**	**24.181 ± 1.302**	**98.50 ± 1.296**

The final experiment results, which are illustrated in **Table 8**, belong to the series of lager fermentations pitched with yeast strain SC3. As it can be seen, they present the total wet and dry yeast crop, viability and total alcohol production after 96h of fermentation and 24h of maturation at 4°C.

The average ethanol percentage in the fermented wort was found to be 6.3% (v/v), with no significant variations in the ABV% values between the three cylinders.

By studying the average total wet yeast yield produced at the end of fermentation, it was found that around 32.5g of yeast slurry was produced, when the desired attenuation level was reached and fermentations were stopped. When, the wet collected yeast crop was dried for a week at 100°C, it was observed that 24g were dry yeast. It also seems that the fermentation which produced the highest percentage of alcohol is also the one with the highest weight of final yeast crop. Thus, it can be assumed that these two fermentation parameters are proportional.

Finally, it was found that only 1.5% of the average number of yeast cells was non-viable.

3.2.1.2 SC3 lager fermentations (Repitching)

This series of fermentations were conducted using the yeast crop produced after completion of the first set of SC3 lager fermentations as inoculum.

3.2.1.2.1 Fermentation profile and sugar utilization

Figure 34 depicts the overall fermentation profile during the static fermentations, which were carried out with the second cycle (repitching) of the lager yeast strain SC3.

The yeast in suspension concentration peaked at $9x10^7$cells/ml 24h into fermentation. Then steadily, cell number in suspension started to decrease due to flocculation, falling to $1x10^7$cells/ml, at the end of fermentation. FAN wort levels commenced to be utilized for the first 43h of the experiment and then they remained constant until the fermentation completion.

Cell viability remained high throughout fermentation, being 97% at the end of the experiment. Cell biomass reached its maximum value during the first 24h of fermentation being 3.6mg/ml of sample and as with cell number in suspension, it started to decline gradually decreasing to 0.9mg/ml of sample after the completion of the fermentation.

Wort gravity decreased normally during fermentation and its target value (3^oPlato) was attained after 96h incubation. The pH, once again, was found to follow a similar decrease profile as FAN, which reduced constantly for the first 43h from the start of the incubation and then remained unaltered until the end of the experiment.

As with the first series of SC3 lager fermentations, the concentration of the fermentable sugars glucose sucrose and fructose reduced gradually during yeast incubation and were entirely removed from the medium after 96h. Maltose showed continuous uptake during the course of fermentation, but its complete uptake did not occur. Similar observations were also observed for maltotriose, but it was found to be nearly completely taken up. As previously discussed (Section ***3.2.1.1.1***), complete uptake of maltotriose and incomplete uptake of maltose is unusual.

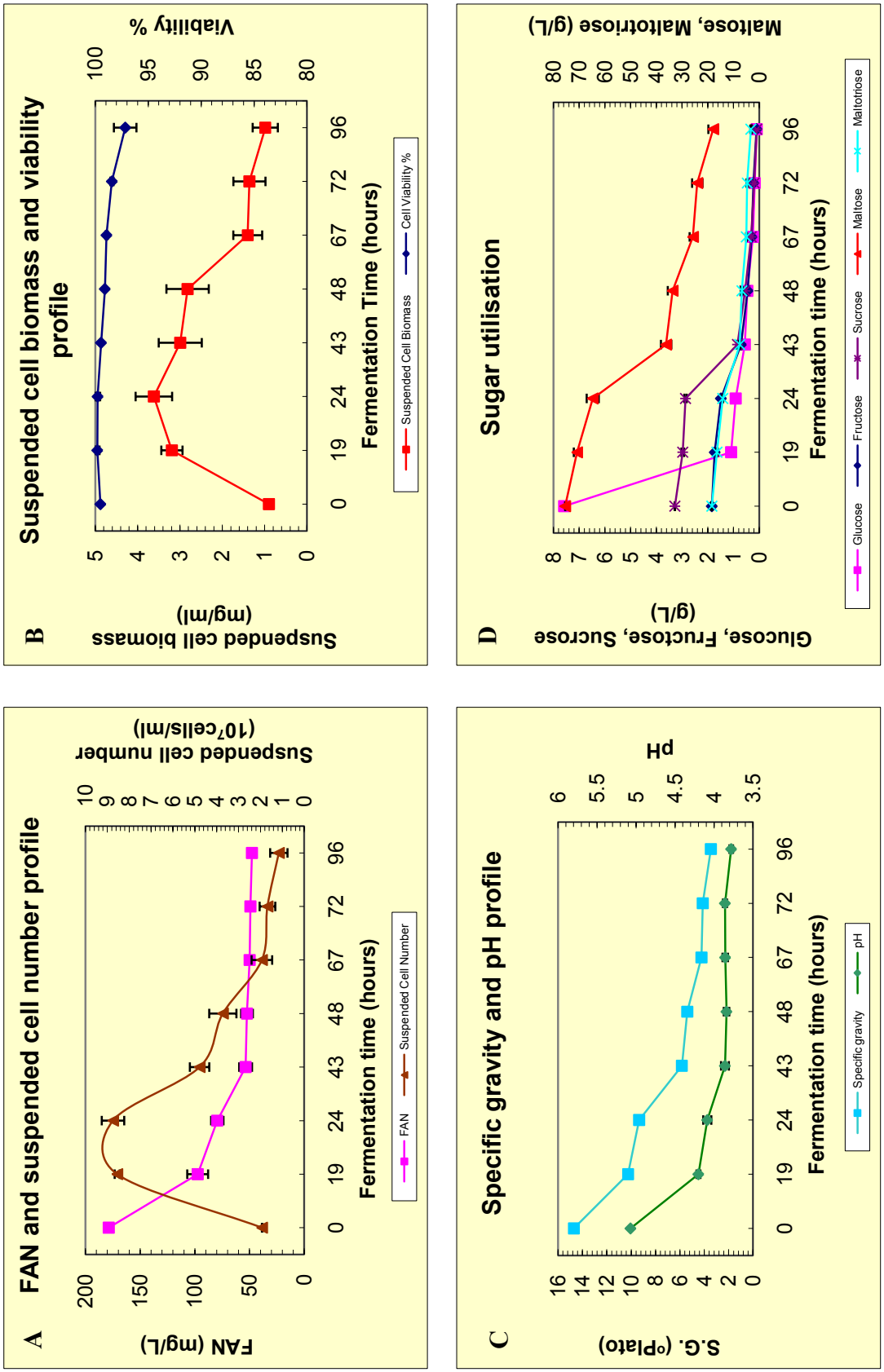


Figure 34: Fermentation profile and sugar utilization for the SC3 lager static fermentations (repitching). The results are the mean values of three fermentations ± S.D.

3.2.1.2.2 Amino acid and ammonia utilization

Amino acid and ammonia uptake for the static fermentations conducted in 2L cylindrical vessels and pitched with the second cycle of the lager yeast strain SC3 are presented in **Figure 35**.

As it was observed, glutamine was the first amino acid of Group 1 utilized by the yeast and was removed from the medium within 19h from the start of the fermentation. Then, the amino acids, whose completed consumption was accomplished 5h after glutamine absorption were glutamate, serine, aspartate, asparagine and lysine. The complete utilization of the two remaining amino acids in this group, arginine and threonine, occurred after 43h fermentation.

In the second group, methionine was absorbed first from the wort after 24h of incubation. Isoleucine was the second amino acid in this group that was successfully consumed by the yeast cells within the next 19h of the experiment. Finally, the rest of the amino acids in Group 2 were totally absorbed after 48h fermentation.

In the third group of amino acids, it can be seen that complete utilization of tryptophan, tyrosine, phenylalanine, glycine and alanine was completed after 48h from the beginning of the experiment.

Proline, did not contribute at all as a nitrogen source, since no absorption of this amino acid took place. On the other hand, ammonia levels decreased very sharply during the first day of the experiment and then reduced steadily until the end of the fermentation with the result that wort ammonia was nearly exhausted at the end.

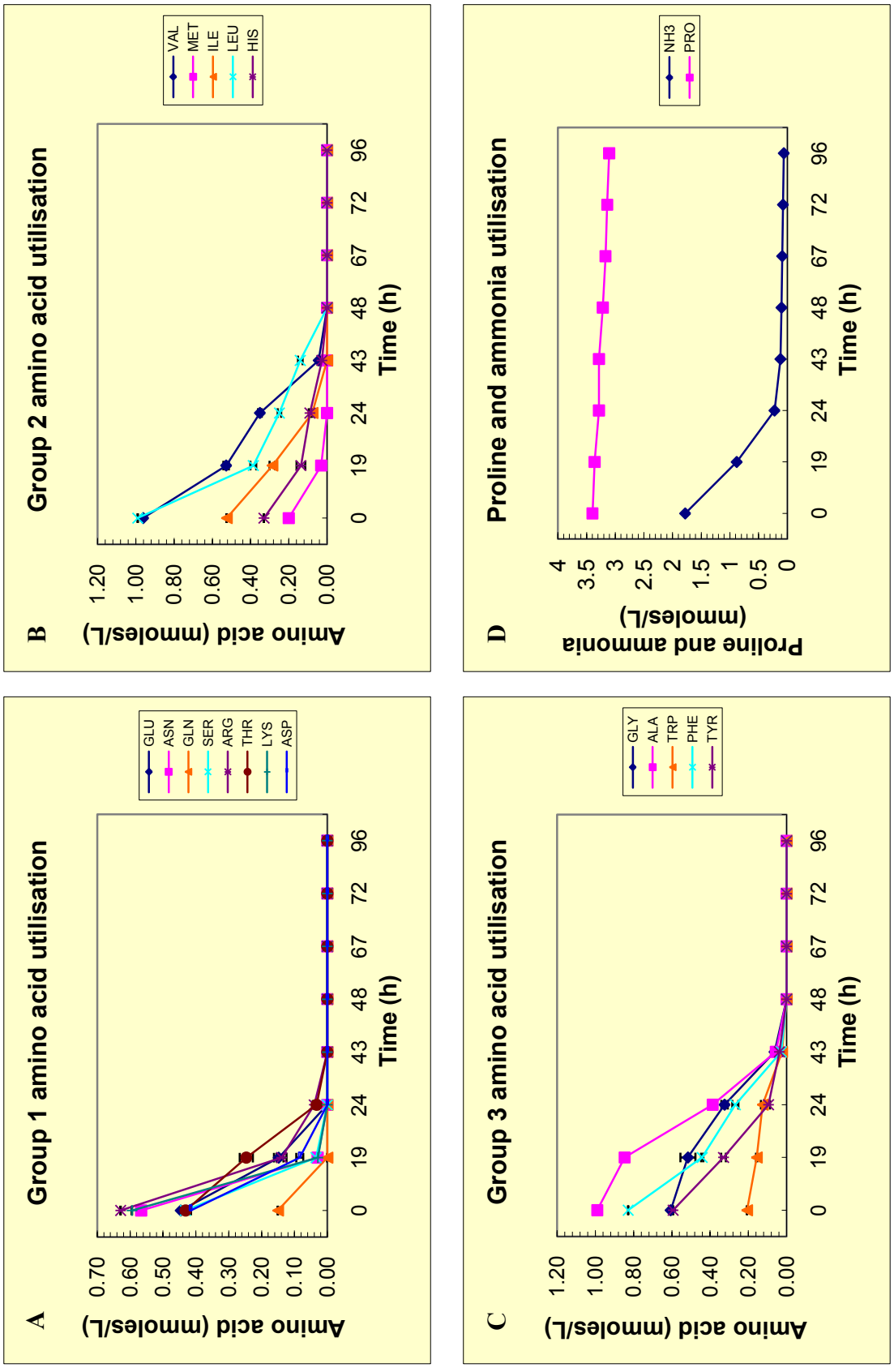


Figure 35: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the SC3 static fermentations (re pitching). The results are the mean values of three fermentations ± S.D.

3.2.1.2.3 Wort nitrogen distribution and nitrogen source utilization

Figure 36 illustrates the detailed nitrogen distribution before and after wort fermentation in terms of the percentage of total wort nitrogen each individual amino acid and ammonia constitutes for the high gravity static fermentations conducted with a 15°Plato + 30% VHM adjunct wort using the second cycle lager yeast strain SC3. The relative nitrogen utilization source is also depicted at in **Figure 36C**.

Amino acids that have been classified in group 1, according to their rate of utilization, constitute only 2.9% of the total nitrogen in unfermented wort. The assimilable nitrogen sources of group 2 were found to represent just 2.4% of the total nitrogen in unfermented wort, whereas group 3 amino acids constitute 3.2% of the total wort nitrogen concentration. After completion of these lager fermentations none of these nitrogen compounds were detected in the fermented wort, meaning that they have all been consumed during incubation. In addition, the most plentiful wort amino acid, proline was found to constitute 3.2% of the total wort nitrogen and 12% of the final fermented wort nitrogen content. Ammonia was estimated to be 11.5% of the initial wort nitrogen concentration, while at the end of fermentation, the part of nitrogen that ammonium ions represented was only 1.5%. This time, the fraction of the total wort nitrogen content that is believed to be oligopeptides was estimated to be 77%.

All the amino acids that represent yeast utilizable nitrogen content were depleted at the end of the fermentation with 100% utilization. Such an observation was not valid for proline, where only 10% of its original concentration was consumed for nitrogen metabolic purposes. As for ammonia, only a negligible proportion (3%) of initial ammonia wort level was left unused at the end of the experiment.

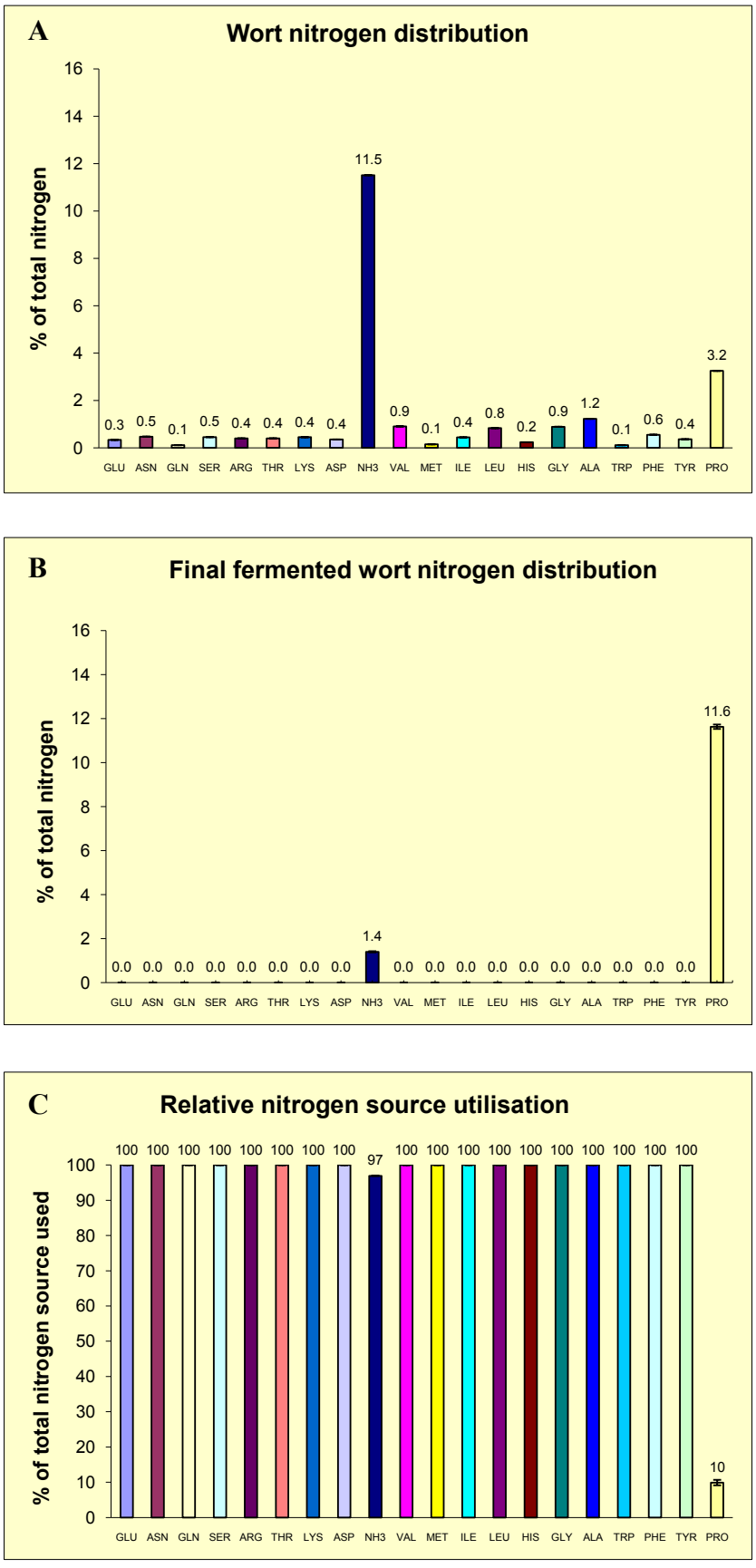


Figure 36: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (SC3 repitched lager static fermentations). The results are the mean values of three fermentations ± S.D.

3.2.1.2.4 Final fermentation measurements

Table 9: Final measurements for **SC3 repitched lager** fermentations (after 96h fermentation at 15°C and incubation at 4°C for 24h)

Parameter / Cylinder	ABV (%)	Total Wet Yeast Biomass (g)	Total Dry Yeast Biomass (g)	Viability (%)
Cylinder 1	6.597	30.637	23.648	96.14
Cylinder 2	6.009	32.855	25.137	98.85
Cylinder 3	6.415	32.977	24.986	100
Average ± S.D.	**6.340 ± 0.301**	**32.156 ± 1.317**	**24.590 ± 0.820**	**98.33 ± 1.982**

Table 9 summarizes the final experimental results for triplicates of lager fermentations, which were inoculated with the yeast crop produced at the end of the SC3 pitched lager fermentations. As shown, there is no difference between the final readings recorded for the first set of fermentations conducted using the SC3 lager strain and the results obtained at the end of the repitched fermentations with this strain.

The mean alcohol concentration of the attenuated wort was measured to be 6.3% (v/v) in both fermentation experiments, where the same percent yeast viability was recorded at the end of both fermentations (98%).

Similar observations were also recorded when the final yeast crop was determined for both sets of fermentation trials. In other words, both the wet and dry yeast crop determinations at the end of the initial and repitched SC3 lager fermentations show no difference to each other.

3.2.1.3 SC4 lager fermentations

3.2.1.3.1 Fermentation profile and sugar utilization

Figure 37 illustrates the metabolic behaviour of the fermentation parameters recorded during the static fermentations carried out with the second industrial lager yeast strain studied, SC4.

Maximum yeast cell number ($7.5x10^7$cells/ml) was obtained 28h from the start of fermentation. Then, cell concentration at 45h decreased to $5x10^7$cells/ml, indicating that yeast cells in suspension commenced to flocculate. At the same time, utilizable nitrogen levels reached their minimum value in the medium after having been used until that sampling point. FAN exhibited a slight increase for the next 60h of fermentation, probably either due to autolysis of the cells or to the proteolytic activity of live cells. After 105h of incubation, FAN levels decreased again until the experiment was completed.

Cell viability started to decline after 45h fermentation, however even after 164h fermentation, cell viability was found to be high (93%). Cell biomass peaked at 3.4mg/ml of sample 28h into fermentation and then its level began to decrease progressively to 0.3mg/ml of sample, after 164h fermentation.

Specific gravity decreased continuously throughout the course of fermentation reaching its target value (2.5^{o}Plato) after 164h. The pH reduced sharply for the first 24h of fermentation and then gradually decreased for a further 26h. Thereafter, its value appeared to increase steadily until the end of the experiment.

The fermentable sugars glucose, sucrose and fructose were completely taken up by the yeast during the first 45h incubation. Maltose exhibited a very sharp decline throughout the whole fermentation duration; however as discussed previously, complete uptake of this sugar was not accomplished. Maltotriose, as occurred during fermentation with the lager strain SC3, performed a continuous utilization during the whole experiment with just traces of this sugar left unconsumed at the end of the fermentation.

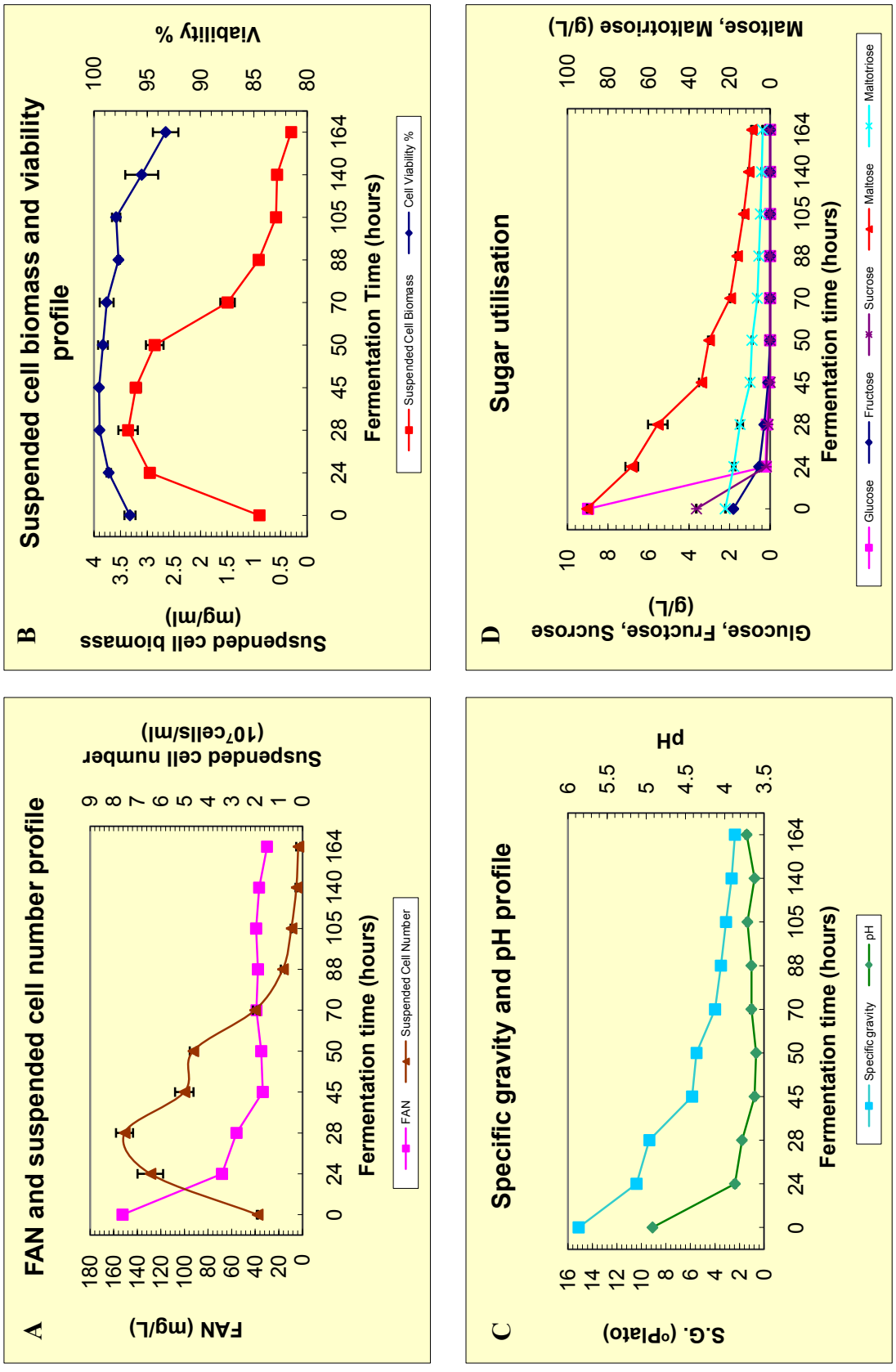


Figure 37: Fermentation profile and sugar utilization for the SC4 lager static fermentations. The results are the mean values of three fermentations ± S.D.

3.2.1.3.2 Amino acid and ammonia utilization

Figure 38 illustrates the nitrogen assimilation of the static fermentations, which were carried out by the industrial lager yeast strain SC4. As shown in the first graph (**Figure 38A**), glutamine was taken up after only 24h of fermentation while the majority of the amino acids of group 1 exhibited also a very speedy consumption, with the result that these were taken up by the yeast cells, after 28h fermentation. Asparagine did not follow the same absorption rate with the rest of the amino acids belonging to group 1, whereas its complete utilization came to an end after a further 17h incubation.

Simultaneously, the amino acids methionine, leucine, isoleucine and histidine, which have been classified in the second absorption group, were also depleted during the first 28h of fermentation, which means that they were also consumed at the same time with the majority of group 1 amino acids. Valine was the only amino acid that constituted an exception to this assimilation pattern, being exhausted after 45h incubation.

When the assimilation rate of the group 3 amino acids was studied, it was found that phenylalanine, tryptophan and tyrosine were totally removed from the fermenting wort at the same time as valine (45h), followed by alanine and glycine, which were depleted after 50h and 70h incubation, respectively.

Finally, it was found that proline's levels commenced to drop from the beginning of fermentation indicating that slow and slight proline utilization was carried out until the end of the experiment. Ammonia wort concentration decreased sharply for the first 45h of the experiment and from then on its levels reduced at a constant rate until all the fermentations ceased, but without totally being consumed.

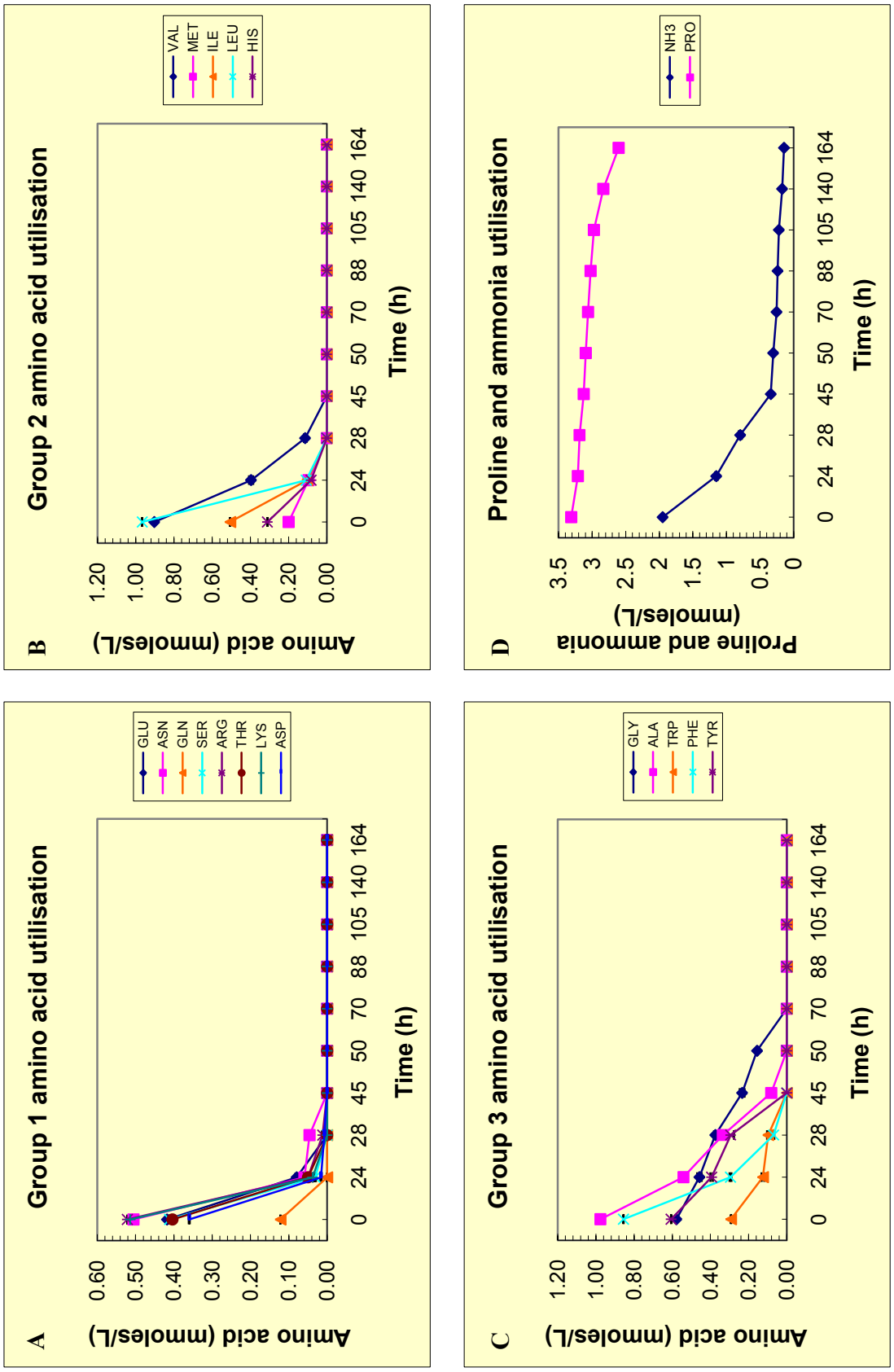


Figure 38: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the SC4 lager static fermentations. The results are the mean values of three fermentations ± S.D.

3.2.1.3.3 Wort nitrogen distribution and nitrogen source utilization

Figure 39 illustrates the detailed nitrogen distribution before and after fermentation in terms of the percentage of total wort nitrogen for each individual amino acid and ammonia, for the high gravity static fermentations conducted with a 15°Plato + 30% VHM adjunct wort using the lager yeast strain SC4. In addition, **Figure 39C** shows the percentage uptake of each wort nitrogen source.

As already discussed, amino acids that have been classified in Groups 1, 2 and 3, according to their rate of utilization, constitute 10% of the total nitrogen in unfermented wort, while ammonia and proline represent 18.5% of the total wort utilizable nitrogen concentration. The residual 71.5% of the overall nitrogen wort content consists of small peptides. On the other hand, at the end of the fermentations, the residual nitrogen materials, which were mainly proline and ammonium ions, represent 21.5% of the remaining free amino nitrogen content unexploited. Similarly, the rest of this nitrogen content portion, which is 78.5%, is considered to comprise unused single peptide substances.

Once again, the degree of utilization of all amino acids was found to be 100%, excluding proline, whose percentage overall consumption was 20%. As for ammonia, only a negligible proportion (7%) of initial ammonia wort levels was left unconsumed at the end of the fermentation.

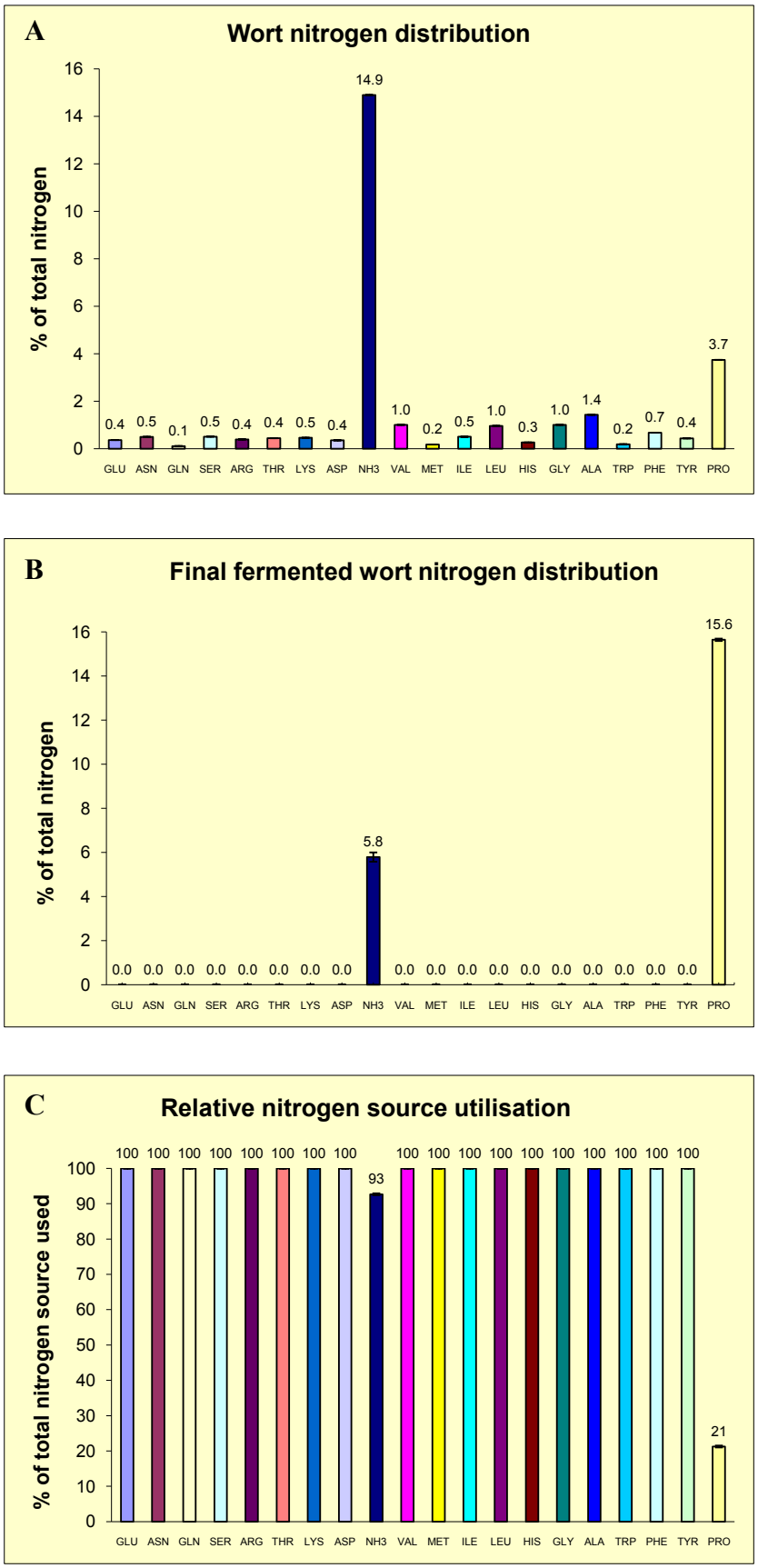


Figure 39: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (SC4 lager static fermentations). The results are the mean values of three fermentations ± S.D.

3.2.1.3.4 Final fermentation measurements

Table 10: Final measurements for **SC4 lager** fermentations
(after 164h fermentation at 15°C and incubation at 4°C for 24h)

Parameter / Cylinder	ABV (%)	Total Wet Yeast Biomass (g)	Total Dry Yeast Biomass (g)	Viability (%)
Cylinder 1	6.766	29.836	20.749	91.17
Cylinder 2	6.605	28.952	19.368	93.36
Cylinder 3	6.827	30.134	21.763	95.30
Average ± S.D.	**6.733 ± 0.115**	**29.637 ± 0.615**	**20.627 ± 1.202**	**93.28 ± 2.066**

The final results of ABV%, total wet and dry yeast crop and cell viability for the first series of fermentations conducted with the lager yeast strain SC4, are shown in **Table 10**.

The average total alcohol level was 6.7% (v/v). The total wet yeast biomass concentration was found to be 29.6g and the dry weight yeast biomass concentration was 9g less than the wet weight results. These 9g were fermented wort, which have evaporated after the slurry samples were placed at 100°C for a week.

The final average cell viability level was 93.3%, meaning that a large number of yeast cells were still viable after 164h fermentation and 24h maturation.

3.2.1.4 SC4 lager fermentations (Repitching)

This series of lager fermentations was carried out by collecting the yeast crop from the first set of SC4 lager fermentations and re-pitching it into a new set of fermentations under the same fermentation conditions.

3.2.1.4.1 Fermentation profile and sugar utilization

Figure 40 shows the changes induced in the fermentation parameters during the static experiments, which were carried out with the second generation of the industrial lager yeast strain SC4.

As with the first set of fermentations, the cell number in suspension obtained its highest value ($8.2x10^7$cells/ml) 28h from the start of fermentation. Thereafter, cell concentration started to decline gradually until the completion of the experiment. FAN wort levels were consumed steadily for 44h and then almost no further utilization of assimilable nitrogen was observed for the remaining 116h of fermentation.

Cell viability was 99.5% for the first 44h of fermentation and then started to decrease progressively falling to 91%, at the end of the experiment. Cell biomass peaked at 3.43mg/ml of sample 28h into fermentation and then its level began to decrease progressively to 0.12mg/ml of sample after 160h of fermentation, as the yeast flocculated out of suspension.

Wort gravity decreased continuously throughout the course of fermentation reaching its target level (2.5^oPlato) after 160h. The pH had fallen to 3.5 after 48h incubation and then it increased to 3.7, at the end of fermentation.

The reducing wort sugars fructose, glucose and sucrose were depleted from the medium after 48h of fermentation. Maltose exhibited a continuous gradual consumption throughout the experiment; however complete utilization of this disaccharide did not occur. Finally, maltotriose was fermented progressively by this lager yeast strain but was not completely taken.

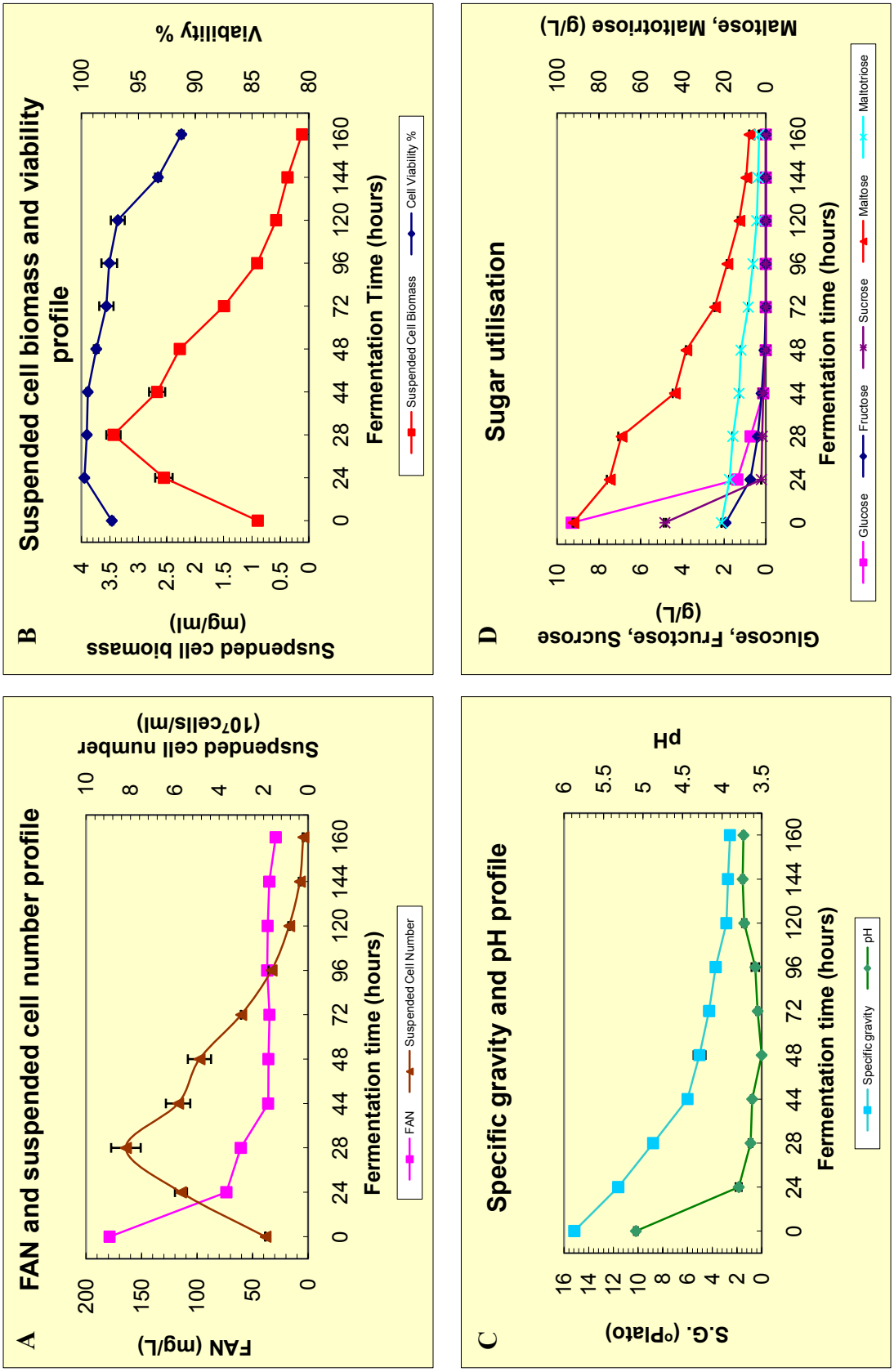


Figure 40: Fermentation profile and sugar utilization for the SC4 lager static fermentations (repitching). The results are the mean values of three fermentations ± S.D.

3.2.1.4.2 Amino acid and ammonia utilization

Figure 41 illustrates the nitrogen assimilation of the static fermentations, which were carried out by the second generation (cycle) of the industrial lager yeast strain SC4.

As shown in **Figure 41A**, the amino acid of Group 1, glutamine exhibited a very rapid consumption performance being taken up by the yeast cells within 24h of fermentation. The remaining six out of seven amino acids (glutamate, serine, threonine, lysine, aspartic acid and arginine) of this group did not follow the same absorption rates and their complete utilization occurred after a further 4h yeast incubation. Finally, complete utilization of the amino acid asparagine followed after 44h.

The majority of the amino acids that have been classified in the second absorption group (**Figure 41B)** were also depleted during the first 28h fermentation. Valine was the sole exception to this assimilation pattern, it being completely taken up at the same time as asparagine (after 44h).

When the assimilation rate of the Group 3 amino acids was studied, it was found that three of the five amino acids of this group had been depleted within 44h of fermentation. In more detail, phenylalanine, tryptophan and tyrosine were totally removed from the fermenting wort after 44h of yeast incubation. As for alanine and glycine, were fully assimilated after 48h and 72h of incubation, respectively.

Finally, proline levels did not reduce significantly, even after 160h incubation, decreasing from 3.35mmoles/L to 2.6mmoles/L. Ammonia wort levels decreased sharply for the first 44h of incubation and from then on its level reduced at a constant linear rate until the fermentations ceased without complete uptake of this nitrogenous wort material.

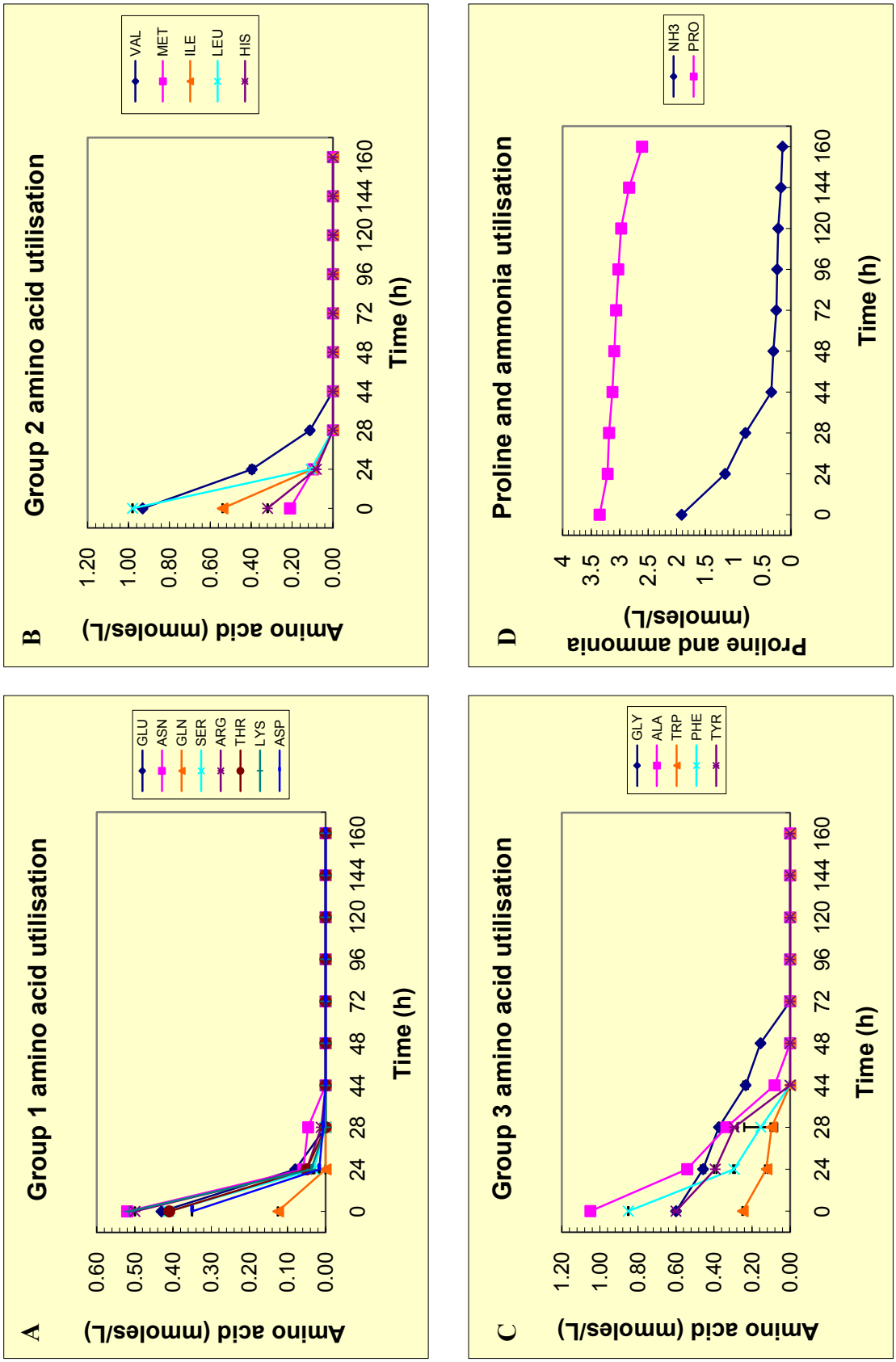


Figure 41: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the SC4 lager static fermentations (repitching). The results are the mean values of three fermentations ± S.D.

3.2.1.4.3 Wort nitrogen distribution and nitrogen source utilization

Figure 42 illustrates the detailed nitrogen distribution before and after fermentation in terms of the percentage of the total wort nitrogen each individual amino acid and ammonia comprises for the high gravity static fermentations conducted with a 15°Plato + 30% VHM adjunct wort using the second generation of the lager yeast strain SC4. The percentage use of each wort available nitrogenous material is shown in **Figure 42C**.

Amino acids that have been classified in Groups 1, 2 and 3, according to their rate of utilization, constitute 9.6% of the total nitrogen in unfermented wort, while ammonia and proline represent 19.5% of the total wort utilizable nitrogen sources. The residual 71.5% of the overall nitrogen wort concentration contains small peptides. On the other hand, at the end of fermentation, the residual nitrogen materials, which were mainly proline and ammonium ions represent 20.1% of the remaining free amino nitrogen content that was left non-metabolized. Similarly, the remainder of this nitrogen content proportion, which is 80% of the total wort assimilable nitrogen, is considered to consist of unused single peptide substances.

Once again, the degree of utilization of all amino acids was found to be 100, excluding proline, whose percentage overall consumption was just 16%. As for ammonia, only a negligible proportion (4%) of initial ammonia wort levels was left in wort at the end of fermentation.

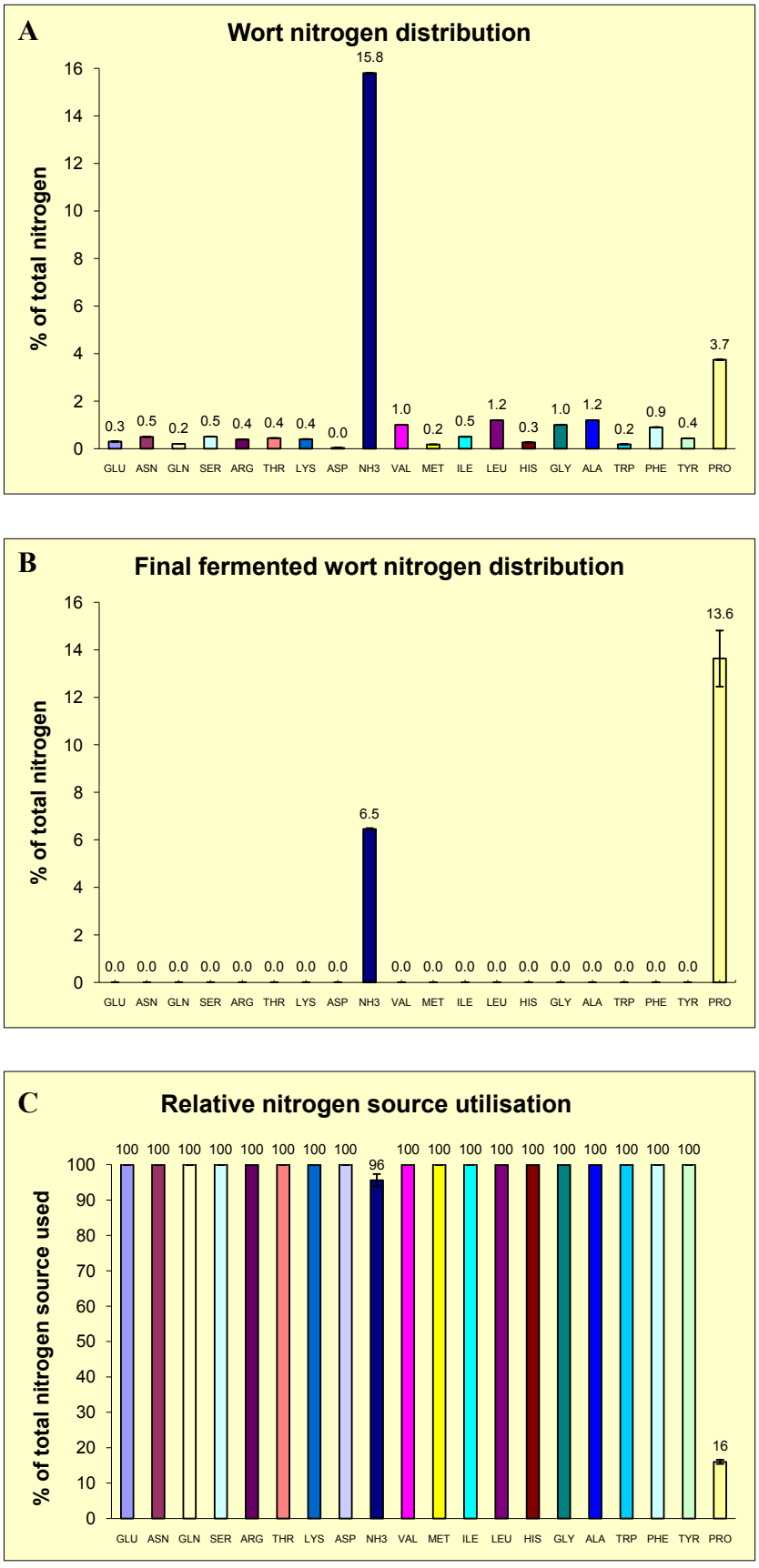


Figure 42: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (SC4 repitched lager static fermentations). The results are the mean values of three fermentations ± S.D.

3.2.1.4.4 Final fermentation measurements

Table 11: Final measurements for **SC4 repitched lager** fermentations (after 160h fermentation at 15°C and incubation at 4°C for 24h)

Parameter / Cylinder	ABV (%)	Total Wet Yeast Biomass (g)	Total Dry Yeast Biomass (g)	Viability (%)
Cylinder 1	6.583	27.924	19.567	92.46
Cylinder 2	6.359	28.312	20.009	95.68
Cylinder 3	6.421	29.722	21.428	93.60
Average ± S.D.	**6.454 ± 0.116**	**28.653 ± 0.946**	**20.335 ± 0.972**	**93.91 ± 1.632**

Table 11 shows the final results measured for the static fermentations pitched with the second generation of the lager yeast strain SC4. As it can be seen, the final ethanol concentration was found to be 6.5% (v/v) and the highest amount was produced in cylinder 1.

The total wet and dry yeast biomass was measured to be 28.7g and 20.3g, respectively. This time, the cylinder with the highest yeast crop was cylinder 3, in which nearly two extra grams of yeast biomass had been formed compared to the first fermentation trial.

The final overall yeast viability was found to be 94% at the end of the 24 hour maturation period. Consequently, no differences were observed with any of the recorded fermentation parameters measured between this set of lager fermentations and the fermentations, which were carried out with the first culture of the same lager strain, after propagation. The only difference that was observed between the first set of fermentations conducted with the lager yeast strain SC4 and the repitched ones with the same yeast strain, was that proline uptake during the repitched series of fermentations reduced by 5%, while ammonia utilization increased by 3%.

3.2.2 Ale fermentations

The two commercial industrial ale yeast strains that Scottish Courage have shown interest for their further experimental exploration and were provided for the completion of our experiments were named **SC5** and **SC8**. As described in the Materials and Methods Section, the same fermentation medium (15^{o}Plato + 30% VHM syrup) was used for all the ale fermentations that were conducted.

3.2.2.1 SC5 ale fermentations

3.2.2.1.1 Fermentation profile and sugar utilization

Figure 43 illustrates the overall fermentation profile of the static fermentations in which the industrial ale yeast strain SC5 was employed. During this series of fermentations, the target gravity (3^{o}Plato) was never achieved and fermentations were terminated after 162h incubation. The same effect was also seen when the yeast crop produced at the end of these fermentations was used for repitching a new series of fermentations. Possible explanations for such an effect are considered in the Discussion Section of this document.

The suspended yeast cell concentration peaked at 6x10^{7}cells/ml, 18h into fermentation. After 24h, the cells flocculated so rapidly that it was difficult to count them. This also had an effect on wort gravity reduction. FAN wort concentrations declined sharply during the first 48h of fermentation and then remained constant until the fermentation was complete.

Cell viability remained at 100% for the first 24h and then started to decline slightly until 66h of fermentation. Thereafter, cell viability declined sharply for the rest of the fermentation; its value at the end of the fermentation was 89%. Cell biomass, as expected, exhibited the same metabolic rate as cell number. Its maximum value was reached within 24h of incubation (2.8mg/ml). Biomass levels dropped noticeably such that hardly any cells remained in suspension.

Wort gravity decreased at a slow rate, with little reduction detected between 42h and 96h fermentation. From then on, only a slight decrease in wort sugar levels was perceived, but even after 162h of incubation, wort gravity was still at 5^{o}Plato, 2^{o}Plato more than the final required gravity level. The pH, once again, was found to have followed a similar pattern as FAN, its value reducing constantly for the first 42h, then remaining unaffected until the end of the experiment.

As shown in **Figure 43D**, the fermentable sugars glucose, sucrose and fructose were all completely utilized at the same time (66h), after gradually being taken up by the yeast cells. In addition, maltose underwent a significant reduction but without being completely absorbed. Finally, maltotriose was not taken up during the fermentation and its initial wort level hardly decreased.

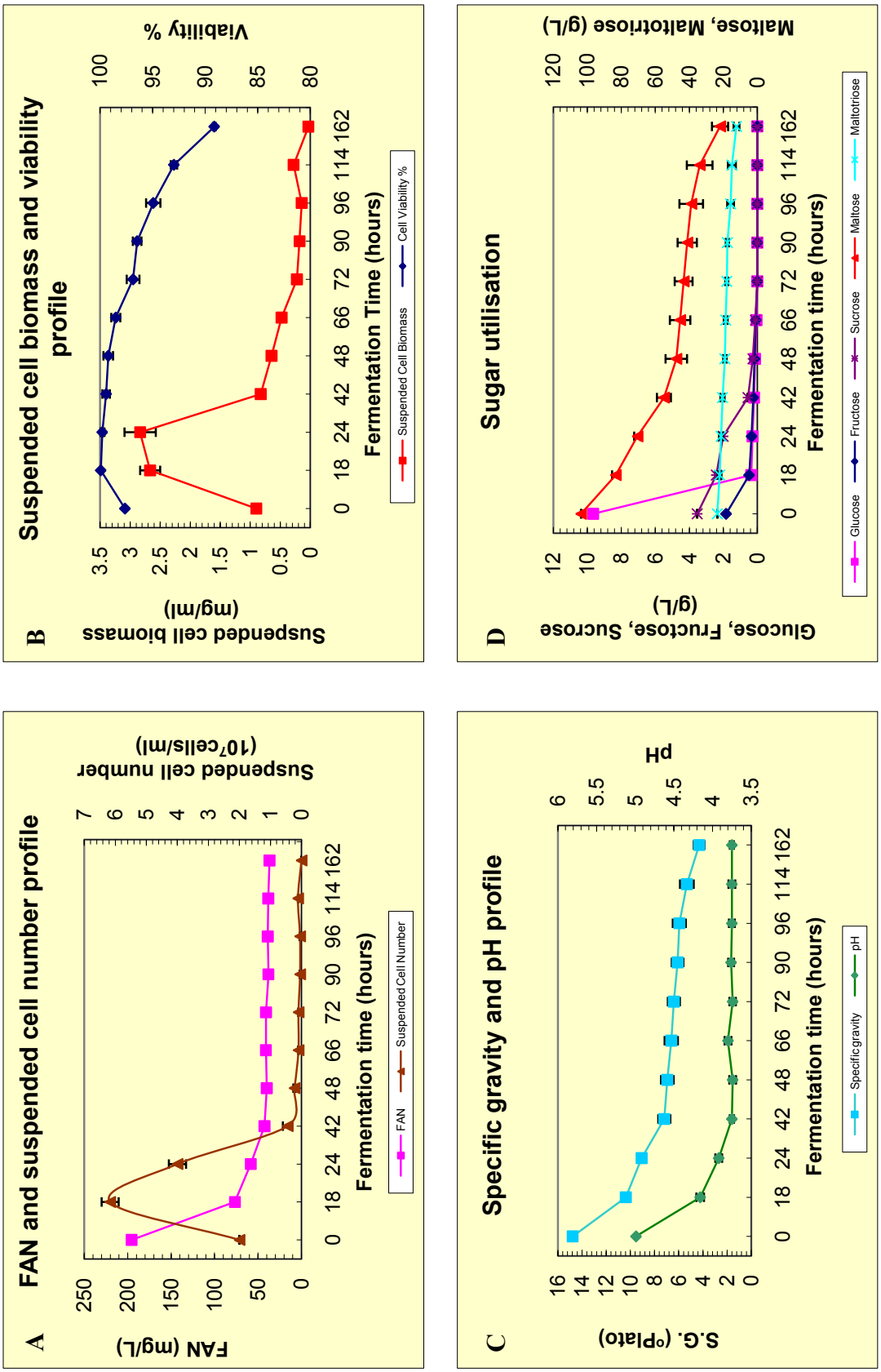


Figure 43: Fermentation profile and sugar utilization for the SC5 ale static fermentations. The results are the mean values of three fermentations ± S.D.

3.2.2.1.2 Amino acid and ammonia utilization

Figure 44 shows the amino acid and ammonia utilization nitrogen during the static wort fermentations conducted with the industrial ale yeast strain SC5.

Amino acids that have been classified in the first group (**Figure 44A**) according to their absorption rate were removed by the yeast during the early stages of fermentation. More precisely, the majority of the amino acids in this group were taken up during the first 24h incubation, excluding glutamine, whose complete removal occurred 6h earlier than other Group 1 amino acids.

In Group 2, methionine also appeared to be totally taken up 18h after yeast pitching, followed by isoleucine and leucine, whose complete absorption occurred after 42h fermentation. Finally the remaining group 2 amino acids, valine and histidine, were removed successfully from the medium during the following 6h of fermentation.

Tryptophan, which has been classified as Group 3 assimilable nitrogen sources (Jones and Pierce, 1964), was taken up 42h from the start of the fermentation, whereas tyrosine, phenylalanine, glycine and alanine were taken up from the fermentation medium 6h later (48h).

Finally, ammonia was absorbed progressively during the first 72h of fermentation and thereafter levels remained almost the same until the completion of the fermentation. Proline, once again, was not preferred as a valuable nitrogenous material by the yeast since its initial level hardly decreased because of the anaerobic conditions.

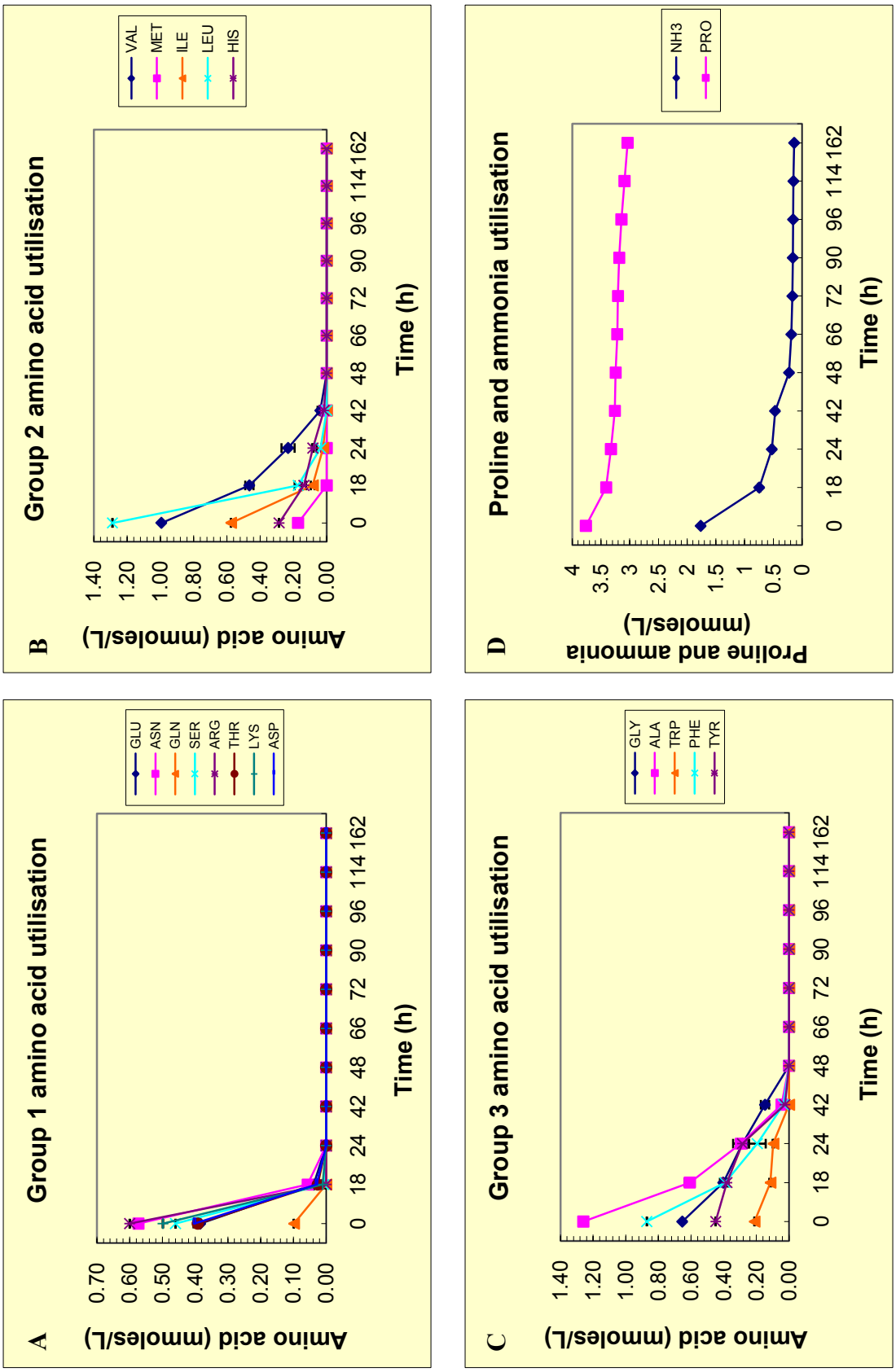


Figure 44: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the SC5 ale static fermentations. The results are the mean values of three fermentations ± S.D.

3.2.2.1.3 Wort nitrogen distribution and nitrogen source utilization

Figure 45 illustrates the detailed nitrogen distribution before and after fermentation in terms of the percentage of total wort nitrogen, each individual amino acid and ammonia constitutes for the high gravity static fermentations conducted with a 15°Plato + 30% VHM adjunct wort using the industrial ale yeast strain SC5. **Figure 45C** shows the percentage utilization of each wort nitrogen source.

The nitrogen sources that are classified in Group 1 constitute only 2.4% of the total nitrogen in unfermented wort and in final fermented wort, none of these nitrogenous compounds was present (**Figure 45B**). In Group 2, which comprises just 2.6% of the total nitrogen in unfermented wort, no residual levels of this group were found in fermented wort. Group 3 amino acids constitute 3.1% of the total wort nitrogen concentration and in fermented wort none of these amino acids were detected in the final nitrogen content. Finally, proline and ammonia constitute almost 3.5% and 10.5% of the total wort nitrogen and 4.5% and 14% of the final fermented wort nitrogen content, respectively. The sum of wort amino acids and ammonia comprise 26.5% of total unfermented wort nitrogen content and the remaining 73.5% is believed to be small peptides that also contribute as nitrogen sources during fermentation.

Ammonia and proline were the only nitrogen compounds that did not exhibit total consumption, whereas 19% of total proline and 92% of total ammonia content were removed by the yeast to contribute its nitrogen nutritional needs.

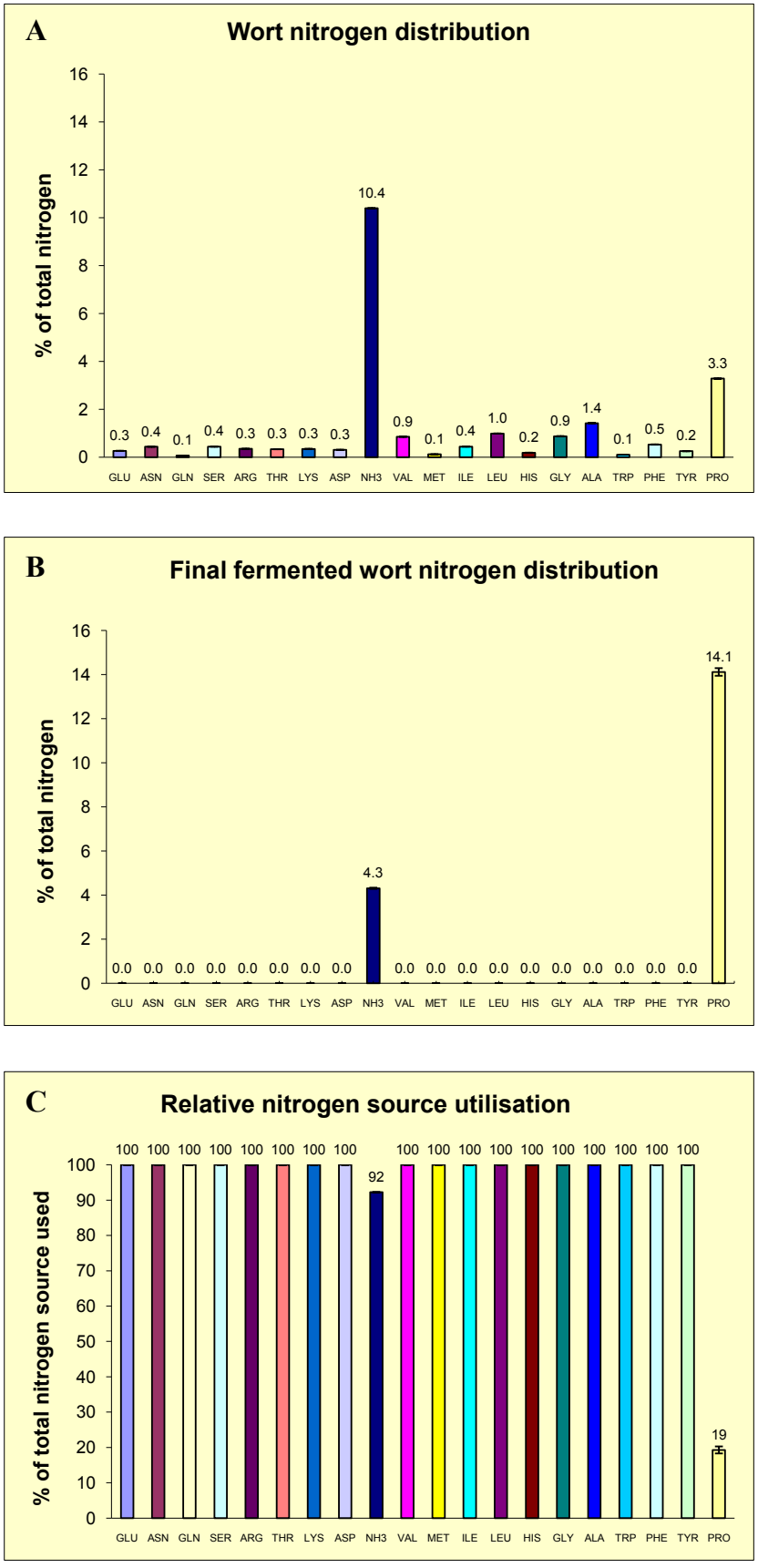


Figure 45: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (SC5 ale static fermentations). The results are the mean values of three fermentations ± S.D.

3.2.2.1.4 Final fermentation measurements

Table 12: Final measurements for **SC5** fermentations
(after 162h fermentation at 18°C and incubation at 4°C for 24h)

Parameter / Cylinder	ABV (%)	Total Wet Yeast Biomass (g)	Total Dry Yeast Biomass (g)	Viability (%)
Cylinder 1	5.805	28.534	20.487	89.22
Cylinder 2	5.927	29.763	20.914	89.52
Cylinder 3	5.825	29.128	21.370	88.40
Average ± S.D.	**5.852 ± 0.065**	**29.142 ± 0.615**	**20.924 ± 0.442**	**89.05 ± 0.580**

The final measurements of the fermented wort with the ale yeast strain SC5 are illustrated in **Table 12**. The total average alcohol concentration was 5.85% (v/v) and the highest percent was noted for the cylinder 2 fermentation. By looking at the final results for the total wet yeast biomass formation, it was found that the average value was 29.14g and the highest amount of yeast was produced again in cylinder 2. In addition, the total average wet biomass after being dried decreased by 8.22g. The final viability of the yeast crop produced was found to be 89%. Once again, the yeast crop produced in the cylinder 2 fermentation vessel was found to have the highest percent of viable cells. Hence, it can be concluded based on the final fermentation measurements, the cylinder 2 fermentation was the most efficient because the highest levels of ethanol and yeast biomass were produced and furthermore, the highest yeast viability proportion was maintained during this fermentation.

3.2.2.2 SC5 ale fermentations (Repitching)

The second series of SC5 ale fermentations commenced immediately following the completion of the first set of ale fermentations. The same fermentation behaviour in terms of sugar attenuation time and yeast flocculation rate was observed during this series of fermentations presuming that the most effective factor for such an effect might have been the geometry of the fermentation vessels. Further possible detailed explanations are considered in the Discussion Section.

3.2.2.2.1 Fermentation profile and sugar utilization

Figure 46 illustrates the overall profile of the fermentation parameters: free amino nitrogen, cell number, biomass and viability, specific gravity and pH during the static fermentations in which the second generation of the industrial ale yeast strain SC5 was used for pitching. As with the first set of fermentations conducted with this ale yeast strain, the fermentations were very sluggish and incomplete. After 168h of yeast incubation, the wort gravity was still 5^{o}Plato, instead of 3^{o}Plato, which was the target gravity (**Figure 46C**). Possible explanations for such a slow yeast performance are considered in the Discussion Section of this document.

The suspended yeast cell concentration peaked at 3.6x10^{7}cells/ml, 24h into fermentation. The rate of yeast sedimentation was rapid, after 66h most of the cells collected at the bottom of the vessel and a very small number remained in suspension. FAN wort levels were utilized for 42h and thereafter they remained almost constant for the rest of the experimental period (**Figure 46A**).

Cell viability maintained 97% for the initial 24h and then it began a sharp decline until the end of the fermentation, when cell viability was 60%. Cell biomass was found to be the highest level (2.2mg/ml of sample) after 24h incubation and at the end of the fermentation was found to have decreased to just 0.15mg/ml.

Wort gravity, as with the first series of fermentations, decreased slowly with result that the target limit attenuation was not achieved even after 168h incubation. Finally, wort pH reduced steadily for the first 42h fermentation and then it remained more or less the same for the rest of the incubation period.

Examining the metabolic behaviour of wort carbohydrates (**Figure 46D**), it can be observed that glucose, sucrose and fructose were depleted after 66h of fermentation. As

for maltose, even though its utilization was continuous, complete absorption of this sugar was not accomplished. Finally, maltotriose was utilized very slowly with the result that high amounts of this sugar were detected in fermented wort.

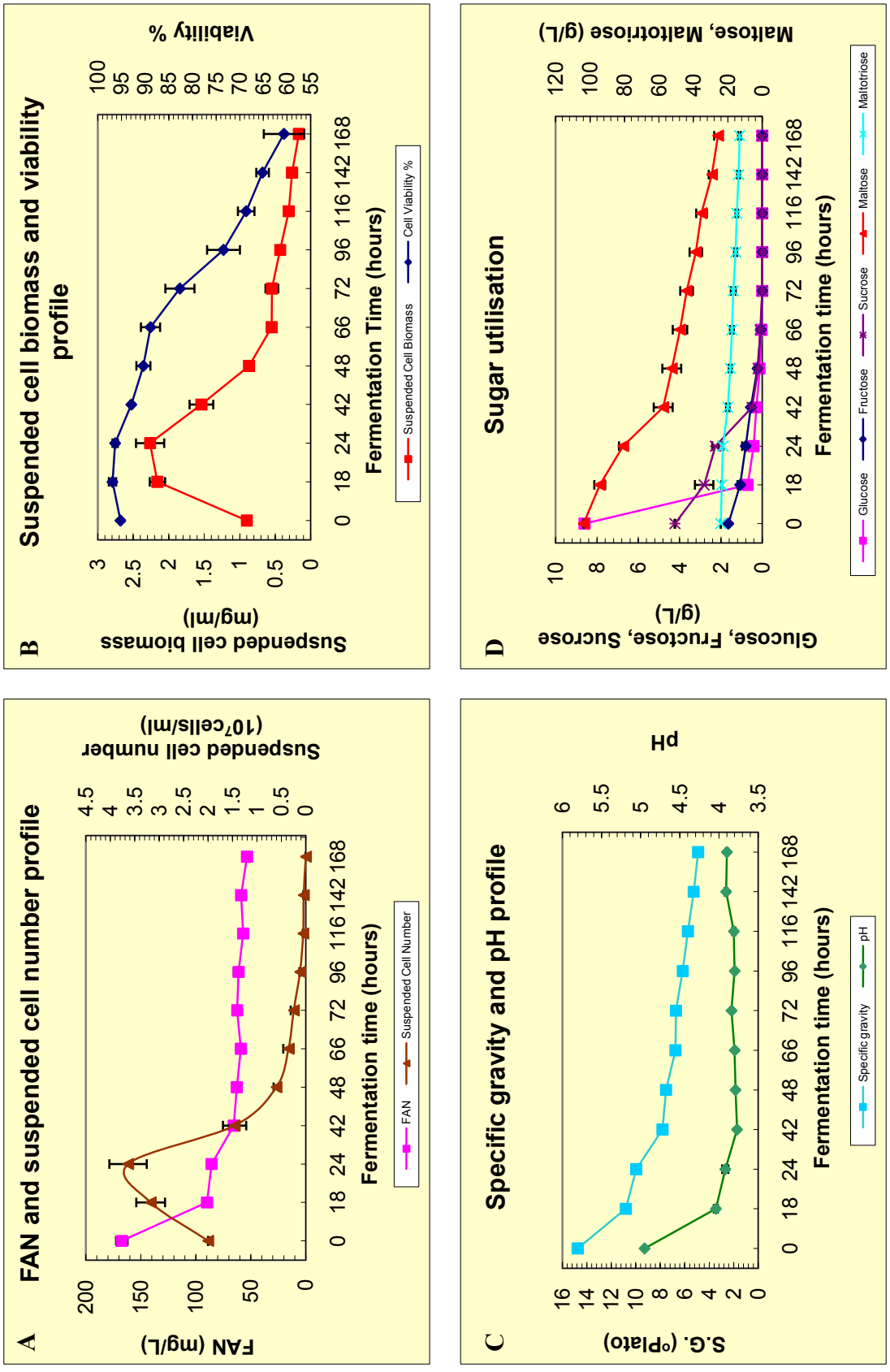


Figure 46: Fermentation profile and sugar utilization for the SC5 ale static fermentations (repitching). The results are the mean values of three fermentations ± S.D.

3.2.2.2.2 Amino acid and ammonia utilization

Figure 47 illustrates the overall nitrogen consumption during the static fermentations conducted with the second cycle of the ale yeast strain SC5.

The amino acids, serine arginine, threonine and lysine belonging to the first group, exhibited very speedy utilization, being completely absorbed during the first 24h fermentation. It should be reported that glutamine was taken up even more rapidly than these amino acids, within 18h from the yeast pitching, while the amino acids glutamic acid and aspartic acid were exhausted after 48h (**Figure 47A**).

The same absorption completion time (18h) with glutamine, was also observed for methionine, regardless of the fact that it is believed to be a Group 2 amino acid. Although, methionine was totally removed from the wort within 18h, isoleucine was depleted after 42h, while the rest of group 2 amino acids achieved complete consumption after 48h of yeast metabolism (**Figure 47B**).

The majority of the amino acids of the third group have undergone a similar utilization pattern compared to the assimilable nitrogen sources of Group 2 amino acids. To be more specific, complete removal of the amino acids tryptophan, tyrosine, phenylalanine and alanine took place after 48h from the start of the experiment, followed by the utilization of glycine, which was totally taken up within the next 18h (**Figure 47C**).

Ammonia again underwent a very sharp reduction during the first 18h of fermentation, followed by a constant reduction pattern, which leaded to its complete utilization after 96h of yeast incubation. As previously observed, proline was not used in this anaerobic static fermentation (**Figure 47D**).

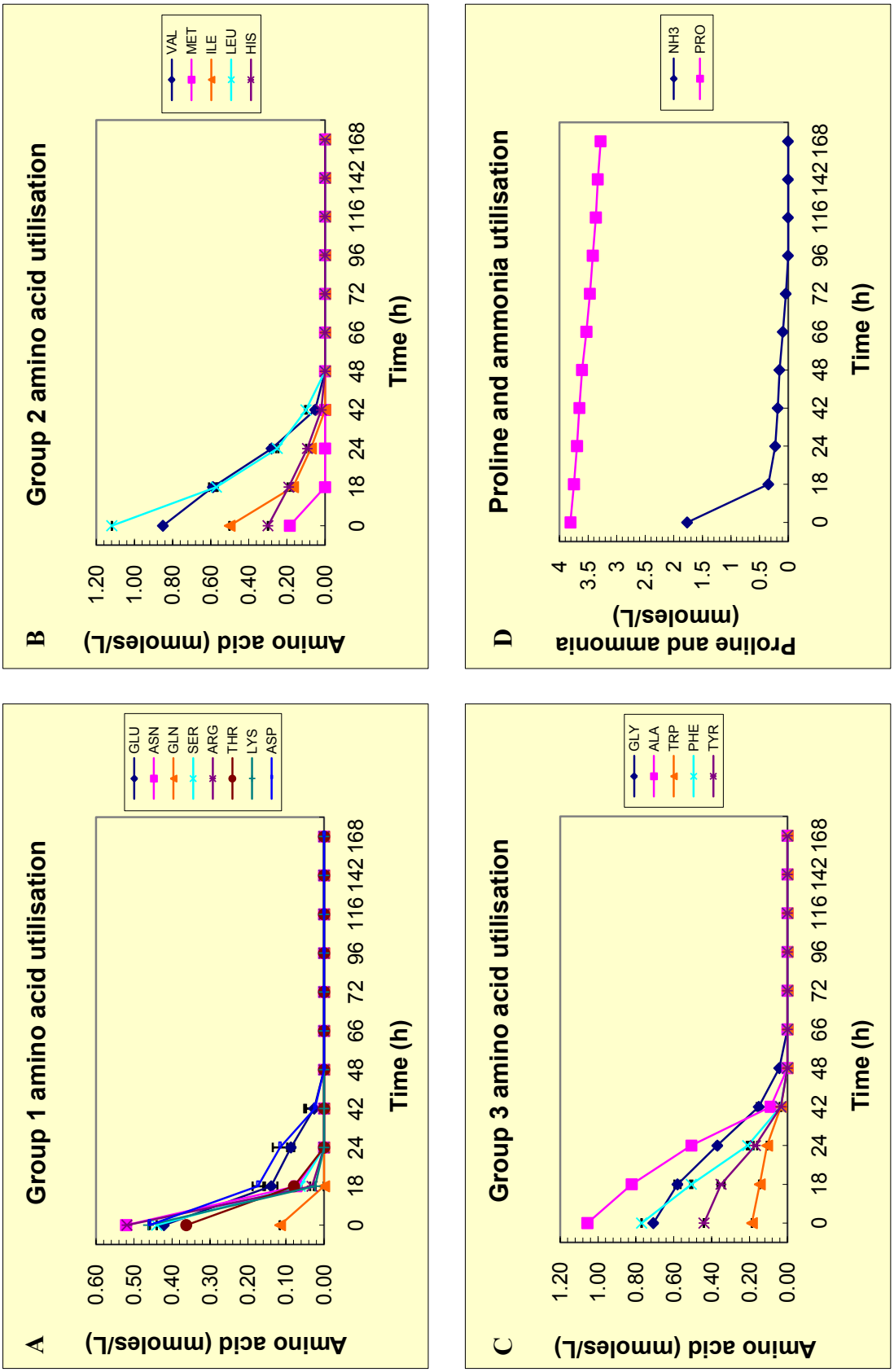


Figure 47: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the SC5 ale static fermentations (repitching). The results are the mean values of three fermentations ± S.D.

3.2.2.2.3 Wort nitrogen distribution and nitrogen source utilization

Figures 48A and B illustrates the detailed nitrogen distribution before and after fermentation in terms of the percentage of total wort nitrogen each individual amino acid and ammonia constitutes for the high gravity static fermentations conducted with a 15°Plato + 30% VHM adjunct wort, using the second generation crop of the ale yeast strain SC5. In addition, the last plot of this figure shows the percentage of utilization of each wort nitrogen source (**Figure 48C**).

The nitrogenous materials that are classified in Group 1 constitute only 2.9% of the total nitrogen in unfermented wort. Group 2 comprises just 2.6% of the total nitrogen in unfermented wort. Group 3 amino acids constitute 3.4% of the total wort nitrogen concentration. In fermented wort the amino acids of these three groups were found to have been completely utilized, thus they do not represent any part of the final fermented wort content. Finally, proline was found to constitute almost 4% of the total wort nitrogen and 10.5% of the final fermented wort nitrogen content. Ammonia was estimated to be 12% of the initial wort nitrogen concentration, while at the end of fermentation did not compose any proportion as part of the remaining nitrogen content. This time, the fraction of the total wort nitrogen content that is believed to be oligopeptides was estimated to be 75%.

All the utilizable yeast nitrogen sources, even ammonia, were found to have been depleted at the end of the incubation period with the only exception being proline; only 14% of its initial wort level was taken up by the yeast cells during fermentation.

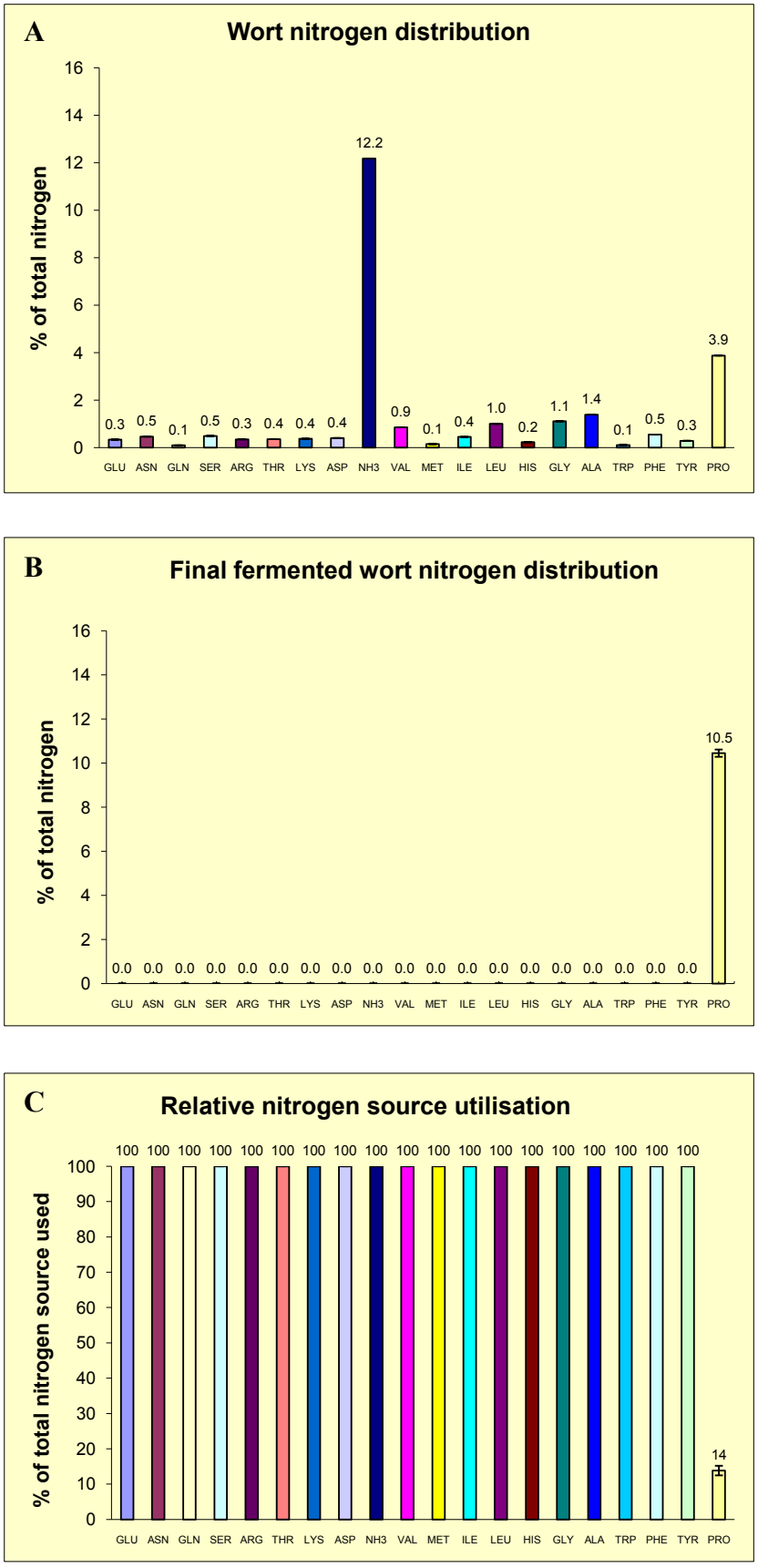


Figure 48: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (SC5 ale repitched static fermentations). The results are the mean values of three fermentations ± S.D.

3.2.2.2.4 Final fermentation measurements

Table 13: Final measurements for **SC5 repitched** fermentations (after 168h fermentation at 18°C and incubation at 4°C for 24h)

Parameter / Cylinder	ABV (%)	Total Wet Yeast Biomass (g)	Total Dry Yeast Biomass (g)	Viability (%)
Cylinder 1	5.573	26.764	18.467	60.71
Cylinder 2	5.971	29.390	21.420	62.53
Cylinder 3	6.008	30.822	22.684	61.60
Average ± S.D.	**5.850 ± 0.241**	**28.992 ± 2.058**	**20.86 ± 2.164**	**61.61 ± 0.910**

The final results of the total wet and dry yeast crop, viability and total alcohol production for the repitched SC5 ale fermentations are illustrated in **Table 13**. The total average ethanol concentration was found to be 5.85% (v/v), exactly the same to that produced after the completion of the initial set of SC5 fermentations. By examining the average total wet yeast crop produced at the end of fermentation, it appeared to be similar to that produced during the first series of experiments. Similarly, such an observation was valid by looking the final value of the yeast crop after drying. In other words, it was found that the final dry yeast weight was 20.9g on both occasions. Nevertheless, when the final yeast viability was measured for the repitched SC5 fermentations, it was realized that it had fallen to 61%, 28% less than the viability found at the end of the first set of fermentations. In terms of yeast and ethanol production, the third fermentations appeared to be more sufficient than the other two; however this fermentation noted the lower viability level recorded from the triplicate experiments. Finally, it is worth mentioning that the ageing factor this time did not seem to have influenced negatively the performance of the second generation of this ale yeast strain with the only exception being the viability parameter.

3.2.2.3 SC8 ale fermentations

3.2.2.3.1 Fermentation profile and sugar utilization

Figure 49 shows the progress of the ale yeast static fermentations inoculated with the industrial ale yeast strain SC8, in terms of FAN, cell suspended concentration (**Figure 49A**), viability and biomass (**Figure 49B**), extract attenuation and pH (**Figure 49C**). In addition the utilization of fermentable sugars is also depicted in the last plot of the figure (**Figure 49D**).

The suspended cell number reached its greatest level ($7x10^7$cells/ml) during the first 24h of fermentation. Then, yeast cells started to flocculate at a steady rate until the end of the experiment. Assimilable nitrogen was used very rapidly for the first 42h of incubation and then its levels were remained more or less the same for the rest of the course of fermentation.

The viability of yeast cells decreased gradually from the start of the experiment until 72h. Then, it exhibited a very sharp decline during the last 42h of fermentation and decreased to 83%. Cell biomass peaked at 3.8mg/ml of sample 24h into fermentation and then its levels began to decrease progressively to 0.25mg/ml of sample after 114h of fermentation.

Wort gravity declined constantly during the course of fermentation. It should be added that the rate of gravity reduction for the first 66h was very rapid and afterwards it continued falling more slowly until its target gravity was achieved. The pH reduced sharply for the first 42h to 3.7 and then it retained this value for 30h. Thereafter, its value appeared to increase slightly to 3.8 until the end of the experiment.

Glucose exhibited a very sharp reduction during the first 18h of fermentation and it was almost depleted, however, its complete utilization was achieved after 72h. Both sucrose and fructose were metabolized gradually during yeast incubation and were also totally consumed after 72h. Maltose exhibited a significant reduction during the first 72h, it falling from 95g/L to 5g/L and thereafter no further consumption of this sugar was observed. Finally, maltotriose was poorly utilized and no significant decrease in its initial level was observed.

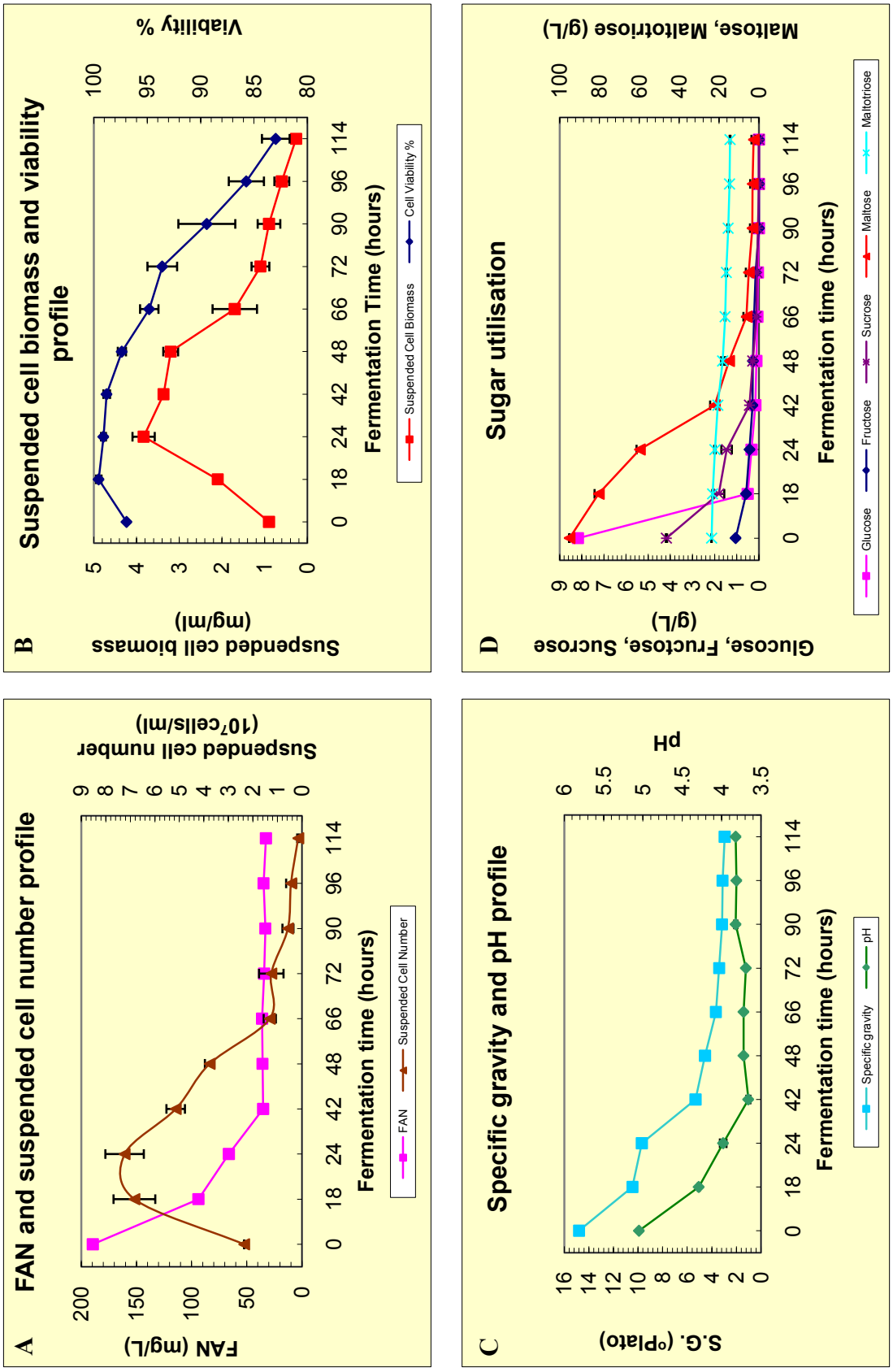


Figure 49: Fermentation profile and sugar utilization for the SC8 ale static fermentations. The results are the mean values of three fermentations ± S.D.

3.2.2.3.2 Amino acid and ammonia utilization

Figure 50 depicts the overall nitrogen utilization during the static fermentations conducted with the industrial ale yeast strain SC8.

As it can be seen in **Figure 50A**, amino acids that have been classified in the first group according to their uptake rate, were removed by the yeast at the early stages of fermentation. The majority of the amino acids of this group were consumed during the first 24h of the experiment, apart from glutamine and arginine, which were completely taken up after 18h and 42h incubation, respectively.

In Group 2 (**Figure 50B**), methionine was the only amino acid that was taken up at the same time as the majority of amino acids of Group 1 (after 24h). The uptake of methionine was followed by complete utilization of the rest of the amino acids belonging to the same group, which were fully utilized 42h into fermentation, excluding histidine, whose total absorption occurred 6h later.

Surprisingly, amino acids of the third group (**Figure 50C**), which according to the literature (Jones and Pierce, 1964) are consumed after the depletion of Group 1 and 2, exhibited nearly the same utilization rate as the Group 2 amino acids. In more detail, tryptophan, tyrosine and phenylalanine were taken up within 42h from the start of the experiment, whereas glycine and alanine disappeared from the fermentation medium 6h later.

Finally, ammonia started being catabolised by the yeast cells from the beginning of fermentation and it exhibited a very sharp decrease between 24h and 42h of fermentation (**Figure 50D**). A slight reduction in ammonia occurred until 72h fermentation and thereafter its concentration remained more or less constant until the end of the incubation. Yeast seemed to utilize proline simultaneously with all of the other nitrogenous sources, but at a very slow rate and to a very low degree with the result that high residual proline levels were found in fermented wort.

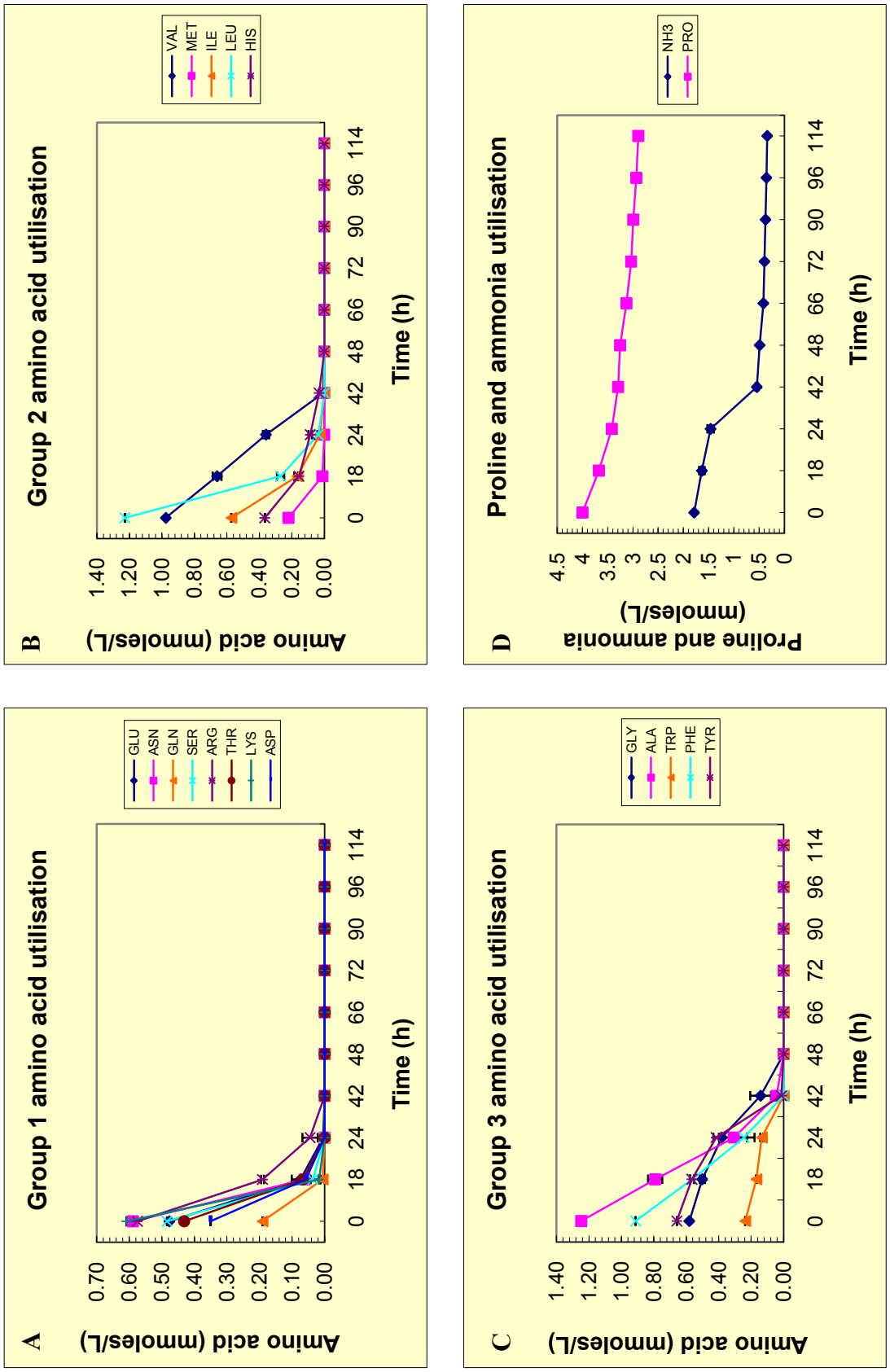


Figure 50: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the SC8 ale static fermentations. The results are the mean values of three fermentations ± S.D.

3.2.2.3.3 Wort nitrogen distribution and nitrogen source utilization

Figure 51 illustrates the detailed nitrogen distribution before and after fermentation in terms of the percentage of the total wort nitrogen each individual amino acid and ammonia constitutes for the high gravity static fermentations conducted with a 15°Plato + 30% VHM adjunct wort by using the industrial ale yeast strain SC8. The last plot (**Figure 51C**) shows the relative nitrogen utilization for every single wort nitrogenous material.

Amino acids that are classified in Group 1 (**Figure 51A**) constitute only 2.8% of the total nitrogen in unfermented wort and 0% in fermented wort (**Figure 51B**), since none of them could be detected at the end of the fermentation. Amino acids of Group 2 comprise the same percentage as Group 1 amino acids of the total wort nitrogen and all of them were efficiently absorbed during fermentation (**Figure 51B**). Group 3 amino acids constitute 3.3% of the total wort nitrogen concentration and in fermented wort do not represent any part of the final nitrogen content, because they have been totally consumed. Finally, proline and ammonia constitute 3.6% and 11% of the total wort nitrogen and 15.5% and 12% of the final wort nitrogen content, respectively. The sum of wort amino acids and ammonia comprises 24% of total unfermented wort nitrogen content and the remaining 76% is believed to be individual small peptides that also contribute as nitrogen sources during fermentation.

As it can be observed during these static ale fermentations, the only nitrogen compounds that have not undergone complete utilization were ammonia and proline. However, as it is shown the percent of the total proline concentration that was consumed during these experiments was high enough (27%) compared to other static fermentations. As for ammonia utilization, 80% of the wort ammonia levels were absorbed during fermentations.

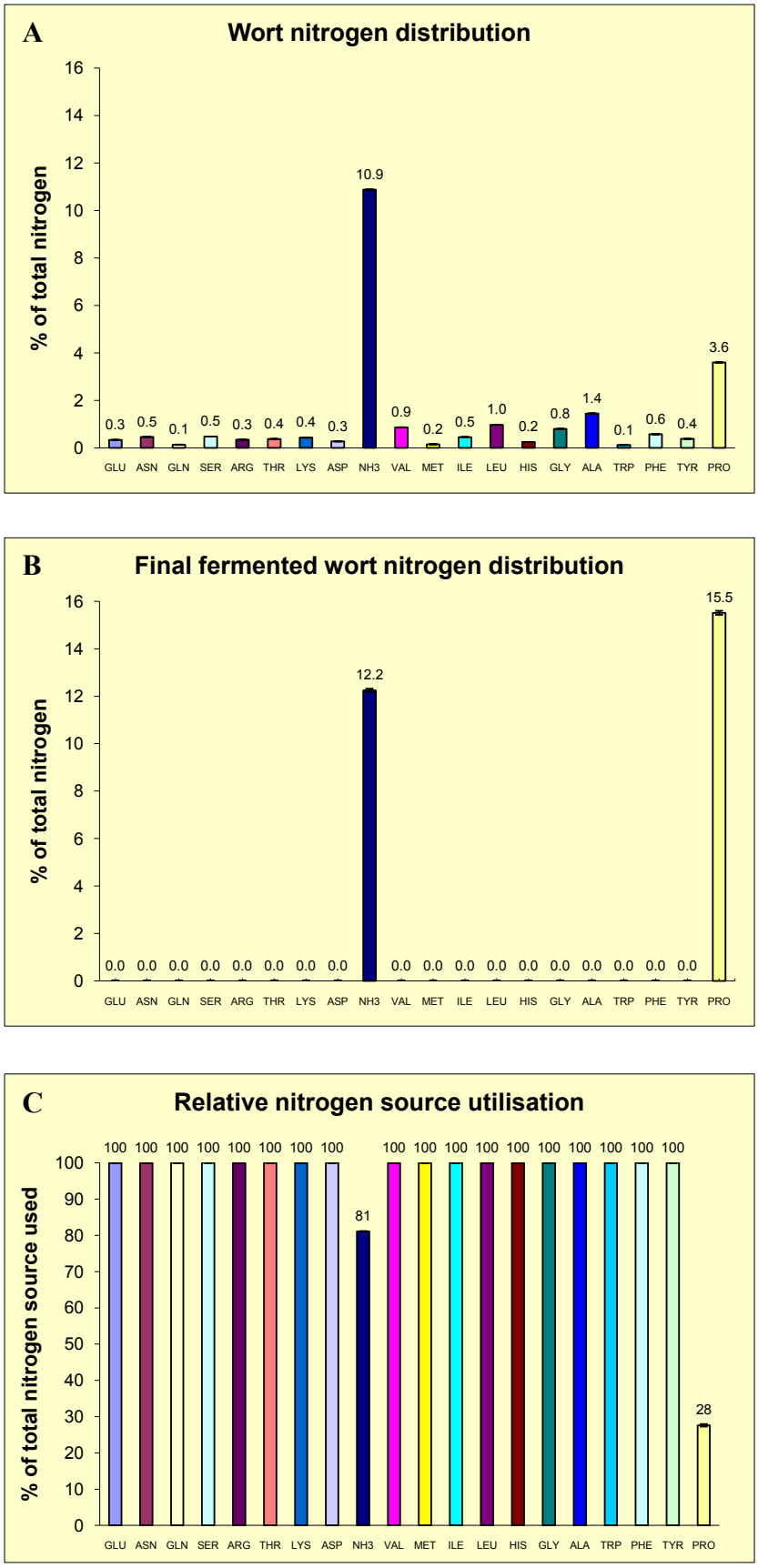


Figure 51: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (SC8 ale static fermentations). The results are the mean values of three fermentations ± S.D.

3.2.2.3.4 Final fermentation measurements

Table 14: Final measurements for **SC8 ale** fermentations
(after 114h fermentation at 18°C and incubation at 4°C for 24h)

Parameter / Cylinder	ABV (%)	Total Wet Yeast Biomass (g)	Total Dry Yeast Biomass (g)	Viability (%)
Cylinder 1	6.573	27.187	19.035	83.09
Cylinder 2	6.419	25.563	17.674	81.22
Cylinder 3	6.861	29.120	20.465	84.01
Average ± S.D.	**6.618 ± 0.224**	**27.290 ± 1.780**	**19.058 ± 1.396**	**82.77 ± 1.422**

Table 14 illustrates the final measurements taken such as ABV%, total wet and dry biomass and yeast viability%, after the completion of the ale SC8 fermentations. The three fermentation cylinders prior to measurements were left at 4°C for 24h in order to induce yeast cells in suspension to settle. The average ethanol concentration was 6.62% (v/v) and cylinder number 3 was the one in which the highest alcohol level was produced. In addition, in the same fermentation vessel, it was found that the highest amount of wet biomass was formed, whereas the total average value of wet biomass for the three fermentations conducted was 27.3g in 1.5l of wort. In continuance, 1.5g of wet yeast biomass from each vessel was placed in pre-weighted aluminum trays, which was left to dry at 100°C for a week. When the trays were re-weighted, it was found that the total average dry biomass was 19.06g. Finally, the viability was measured and it was found that it had decreased by 2.2% from the last sampling point, decreasing to 82.8%. Cylinder 3 was the one that exhibited the best fermentation performance in terms of highest ethanol and biomass production and moreover exhibited the highest viability percentage of yeast cells.

3.2.2.4 SC8 ale fermentations (Repitching)

3.2.2.4.1 Fermentation profile and sugar utilization

Figure 52 illustrates the profile of fermentation parameters measured during the static fermentations, which were conducted with the second generation culture of the industrial ale yeast strain SC8. The time taken in order to reach full attenuation was 96h.

Maximum number of cells in suspension was achieved during the first 24h of fermentation ($6.7x10^7$cells/ml) (**Figure 52A**), whereas thereafter yeast cells began to sediment. FAN wort levels started to be used rapidly from the beginning of fermentation and after 44h, FAN concentration remained constant until the end of the experiment.

Cell viability underwent a significant change during the first 24h of fermentation, increasing from 83% to 98%. Then it started to decrease gradually and after 96h of fermentation decreased to 86%. Cell biomass reached its maximum value after 24h of incubation (3.6mg/ml of sample) and then cell levels began to reduce until the end of fermentation (**Figure 52B**).

Specific gravity underwent a gradual reduction during 68h of fermentation. Between 68h and 72h of fermentation, wort gravity levels remained unchanged and then in the last 24h, a very slight decrease was observed. Finally, wort pH followed a similar reduction pattern as FAN, where it reduced sharply for the first 44h and then remained more or less unchanged until the end of the fermentation (**Figure 52C**).

The wort fermentable sugars, glucose, fructose and sucrose depleted after 72h of fermentation. Maltose followed a sharp reduction for the first 68h incubation and thereafter its levels remained nearly at the same value until the fermentation completion. Finally, maltotriose did not show any significant decrease during the whole course of fermentation and its concentration remained more or less unchanged (**Figure 52D**).

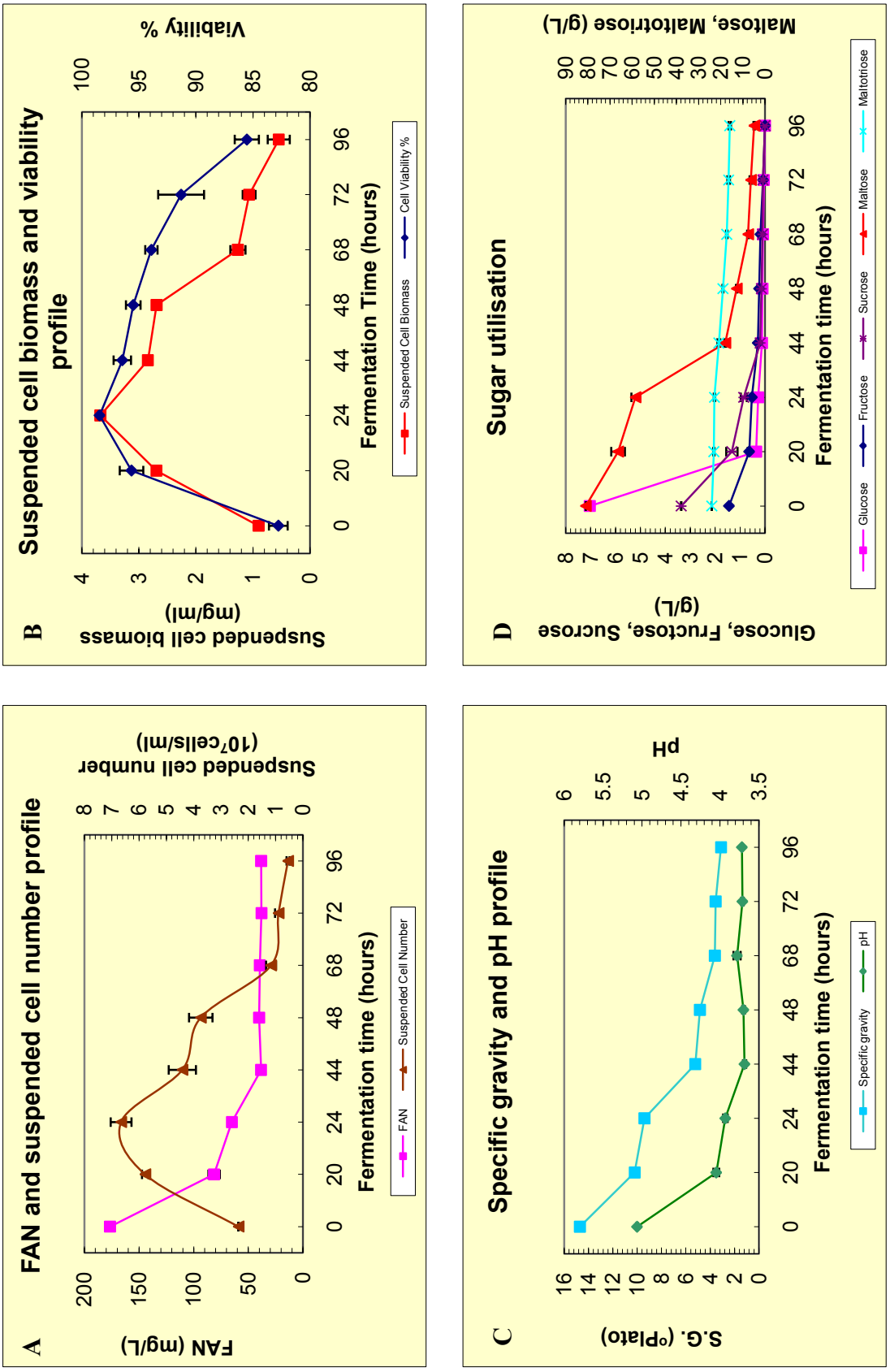


Figure 52: Fermentation profile and sugar utilization for the SC8 ale static fermentations (repitching). The results are the mean values of three fermentations ± S.D.

3.2.2.4.2 Amino acid and ammonia utilization

The general amino acid and ammonia uptake that for the SC8 repitched ale fermentations, is presented in **Figure 53**. This data was obtained by using the yeast crop collected after the first series of fermentations with the SC8 ale yeast strain was complete. The amino acid glutamine of the first group disappeared from the medium within the first 20h of fermentation (**Figure 53A**). Four hours later, the amino acids serine, aspartate, lysine, asparagine and glutamate were selected by the yeast to be metabolized and arginine and threonine were the last two amino acids of this group which were fully utilized within 44h of fermentation.

In addition, methionine (in Group 2) was also taken up by the yeast cells at the same time as glutamine (after 20h). The rest of the amino acids belonging to this group, apart from valine and histidine, were depleted at the same time as arginine and threonine. Valine and histidine were exhausted four hours after the complete uptake of the other amino acids (**Figure 53B**).

Tryptophan and phenylalanine are group 3 amino acids, which were also removed from the wort within 44h of fermentation and then tyrosine, glycine and alanine eventually were utilized by yeast and no traces of these two amino acids could be detected after 48h fermentation (**Figure 53C**).

Ammonia wort levels declined sharply for the first 24h of fermentation and then they continued to reduce in step until the end of fermentation but without all the ammonia to be entirely taken up by the yeast. Proline showed a very slight utilization for the first 44h of fermentation but thereafter its concentration remained more or less constant until the end of the fermentation.

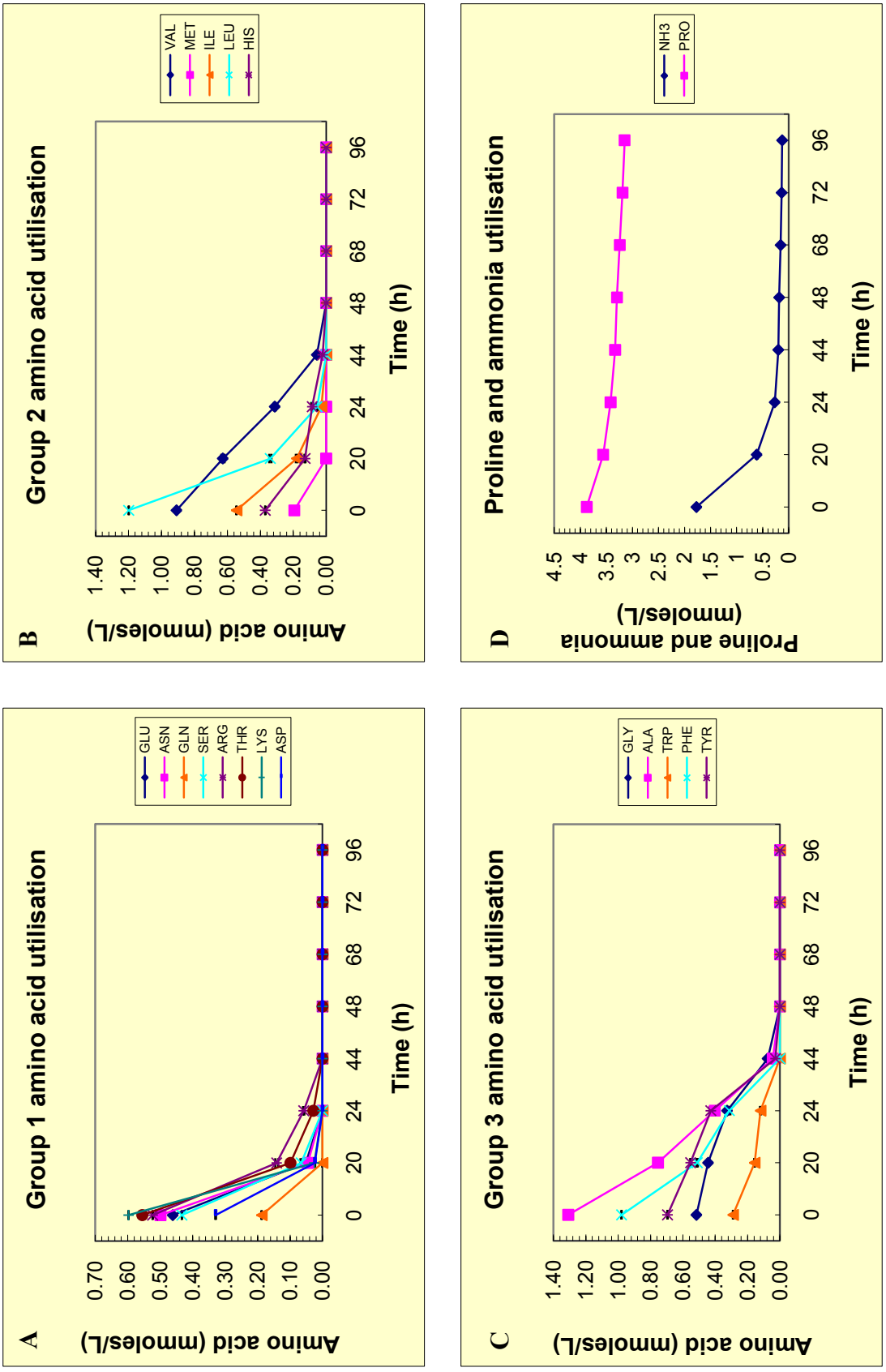


Figure 53: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the SC8 ale fermentations (repitching). The results are the mean values of three fermentations ± S.D.

3.2.2.4.3 Wort nitrogen distribution and nitrogen source utilization

Figure 54 illustrates the detailed nitrogen distribution before and after fermentation in terms of the percentage of total wort nitrogen, each individual amino acid and ammonia constitutes for the high gravity static fermentations conducted with a 15°Plato + 30% VHM adjunct wort by using the second generation (cycle) of the industrial ale yeast strain SC8. **Figure 54C** shows the relative nitrogen utilization for every single wort nitrogenous material.

Amino acids that are classified in the Group 1 constitute only 1.4% of the total nitrogen in unfermented wort and are not present in final fermented wort. Amino acids of Group 2 comprise just 2.8% of the total nitrogen in unfermented wort and no part of the fermented wort nitrogen content. Group 3 amino acids constitute 4% of the total wort nitrogen concentration and in fermented wort they do not represent any part of the final nitrogen content as they have been totally consumed. Finally, proline and ammonia constitute almost 4% and 12% of the total wort nitrogen and 13.5% and 4% of the final fermented wort nitrogen content, respectively. Amino acids and ammonia comprise 24% of total unfermented wort nitrogen content and the remaining 76% is believed to be single small peptides that also contribute as nitrogen sources during fermentation.

The only nitrogen compounds that have not undergone complete utilization were ammonia and proline. However, the percentage of the total proline concentration that was consumed during these experiments was high (19%) compared to that used during shake flask fermentations. As for ammonia utilization, 93% of the wort ammonia levels was absorbed during fermentation.

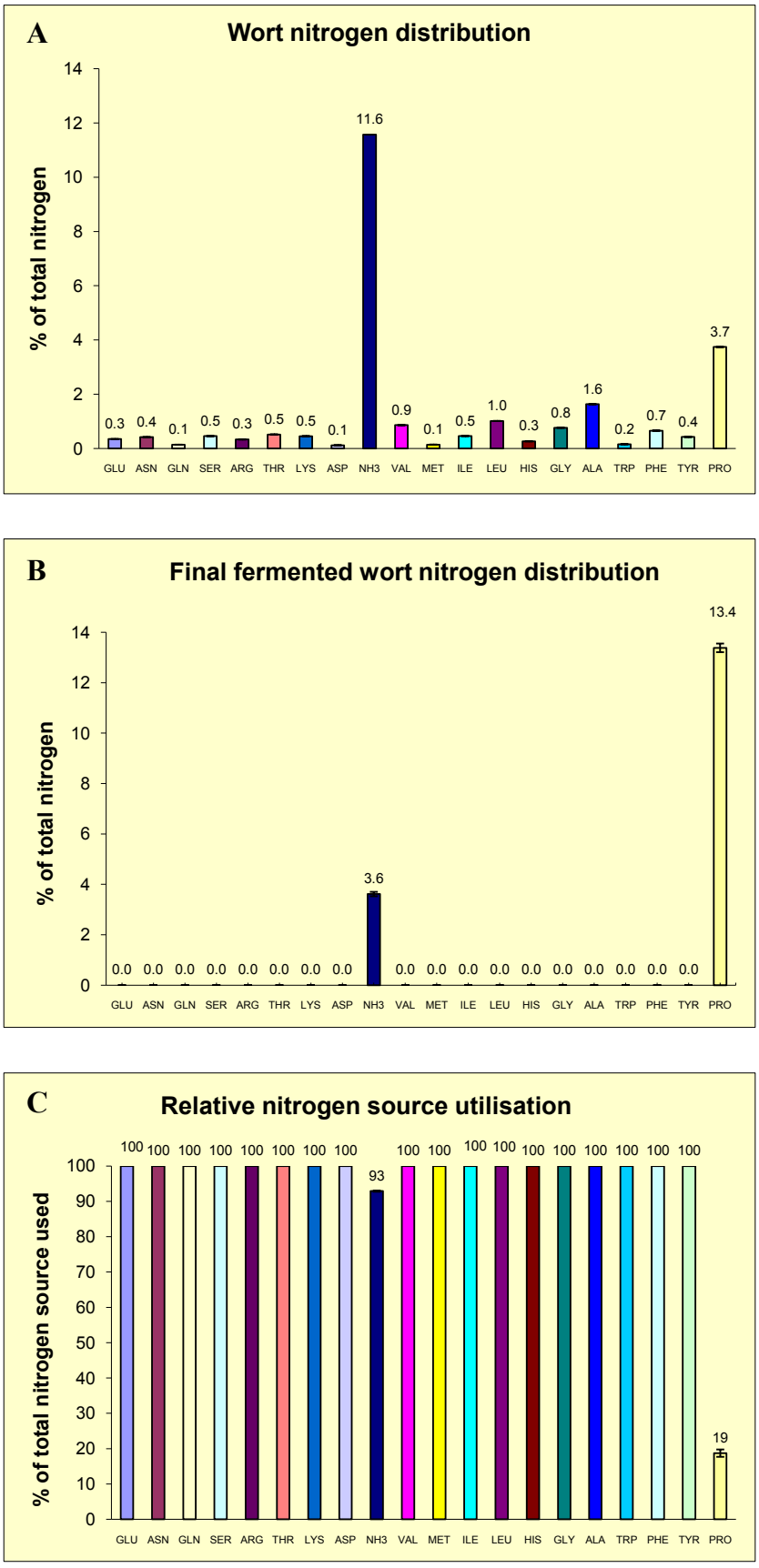


Figure 54: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (SC8 ale static repitched fermentations). The results are the mean values of three fermentations ± S.D.

3.2.2.4.4 Final fermentation measurements

Table 15: Final measurements for **SC8 repitched ale** fermentations (after 96h fermentation at 18°C and incubation at 4°C for 24h)

Parameter / Cylinder	ABV (%)	Total Wet Yeast Biomass (g)	Total Dry Yeast Biomass (g)	Viability (%)
Cylinder 1	6.895	26.764	19.213	84.15
Cylinder 2	6.770	29.390	21.105	82.38
Cylinder 3	6.992	30.822	22.138	85.47
Average ± S.D.	**6.886 ± 0.111**	**28.992 ± 2.058**	**20.819 ± 1.483**	**84.00 ± 1.550**

Table 15 shows the final measurements taken (alcohol levels, total wet and dry biomass and viability %) for the complete ale SC8 repitched fermentations, after their incubation at 4°C for 24h. The total average ethanol concentration was 6.89% (v/v), slightly higher than the final ethanol percentage formed at the end of the first series of fermentations conducted with the same yeast strain. Once again in cylinder number 3, the highest alcohol level was produced. The flocculated wet biomass produced after sedimentation of the suspended yeast cells was higher than that produced during the fermentations, pitched with the first generation of the ale yeast strain. Such an effect was also reflected in the total dry biomass, which was found to be higher by approximately 1.8g than that produced during the first series of SC8 fermentations. The third fermentation was also the one that produced the most elevated total biomass level. The viability was found to have decreased by 2% (to 84%) from the measurement taken after 96h yeast incubation.

3.3 Fermentation supplementations

A series of wort supplementation experiments were conducted, wort was supplemented with two and five times their natural wort concentration of the amino acids L-lysine, L-methionine, L-arginine and with just twice the natural wort concentration of ammonia. These amino acids were selected to be the nitrogen wort supplements since during shake flask fermentations they were the amino acids preferred by yeast cells. In other words, they were the only amino acids that were completely utilized (100%) during shake flask trials regardless of the fermentation medium used. Ammonia was used as an additional wort nitrogen supplement, since it is a readily used inorganic nitrogen source for *Saccharomyces cerevisiae*.

The supplementation experiments were conducted in order to investigate the contribution effect of these wort nitrogen supplements on the time required for yeast to achieve the complete fermentation sugar attenuation level. In other words, if their wort concentration increase favors or inhibits the yeast fermentation ability. The yeast strain used for all these experiments was the lager strain SC3 since according to the static fermentation results this strain exhibited the most healthy and efficient fermentation metabolic activity. Finally, it should be noted that only the five times amino acid supplementation results are presented in this section since no significant difference was observed between the two and five times supplementations.

The lysine-supplemented fermentations were considerably faster (completed in 48h) than the control fermentations (96h). Similar effects were recorded with fermentations supplemented with arginine (completed in 67h). Supplementation of wort with methionine had an inhibitory effect on yeast fermentation (completed in 103h). Similar observations were obtained when wort was supplemented with ammonia (212h to completion).

3.3.1 Control fermentations

3.3.1.1 Fermentation profile and sugar utilization

Figure 55 shows the profile of the fermentation parameters during the 2L static control-unsupplemented experiments, which were carried out with the lager yeast strain SC3. The control fermentation results are the average for all the unsupplemented fermentations conducted simultaneously with every single amino acid supplementation experiment, for comparison purposes. Fermentations were found to require 96h in order for full attenuation to be achieved.

With the first series of fermentations pitched with the strain SC3, the yeast cell number in suspension obtained its highest value ($10x10^7$cells/ml) 24h from the start of fermentation. Thereafter, cell concentrations reduced gradually until the completion of the experiment. FAN wort levels were consumed steadily for 43h and then very insignificant fluctuations occurred in FAN value, meaning that no further utilization of assimilable nitrogen was observed for the rest of the incubation period (**Figure 55A**).

Cell viability was found to be high for the course of the fermentation decreasing to 98% at the end of the experiment. Cell biomass peaked at 3mg/ml 24h into fermentation and then its levels began to decrease progressively to 0.7mg/ml after 96h of fermentation (**Figure 55B**).

Wort gravity decreased continuously throughout the course of fermentation reaching its target level (3^oPlato) after 96h (**Figure 55C**). The pH followed a downward course throughout the fermentation, it falling to 3.6 from 5.1, after 96h incubation.

The wort sugars sucrose, fructose and glucose were depleted at the end of the experiment after 96h. Maltotriose was used rapidly during yeast incubation, with traces left unfermented. Maltose was consumed gradually after 24h, but incomplete attenuation of this sugar occurred (**Figure 55D**).

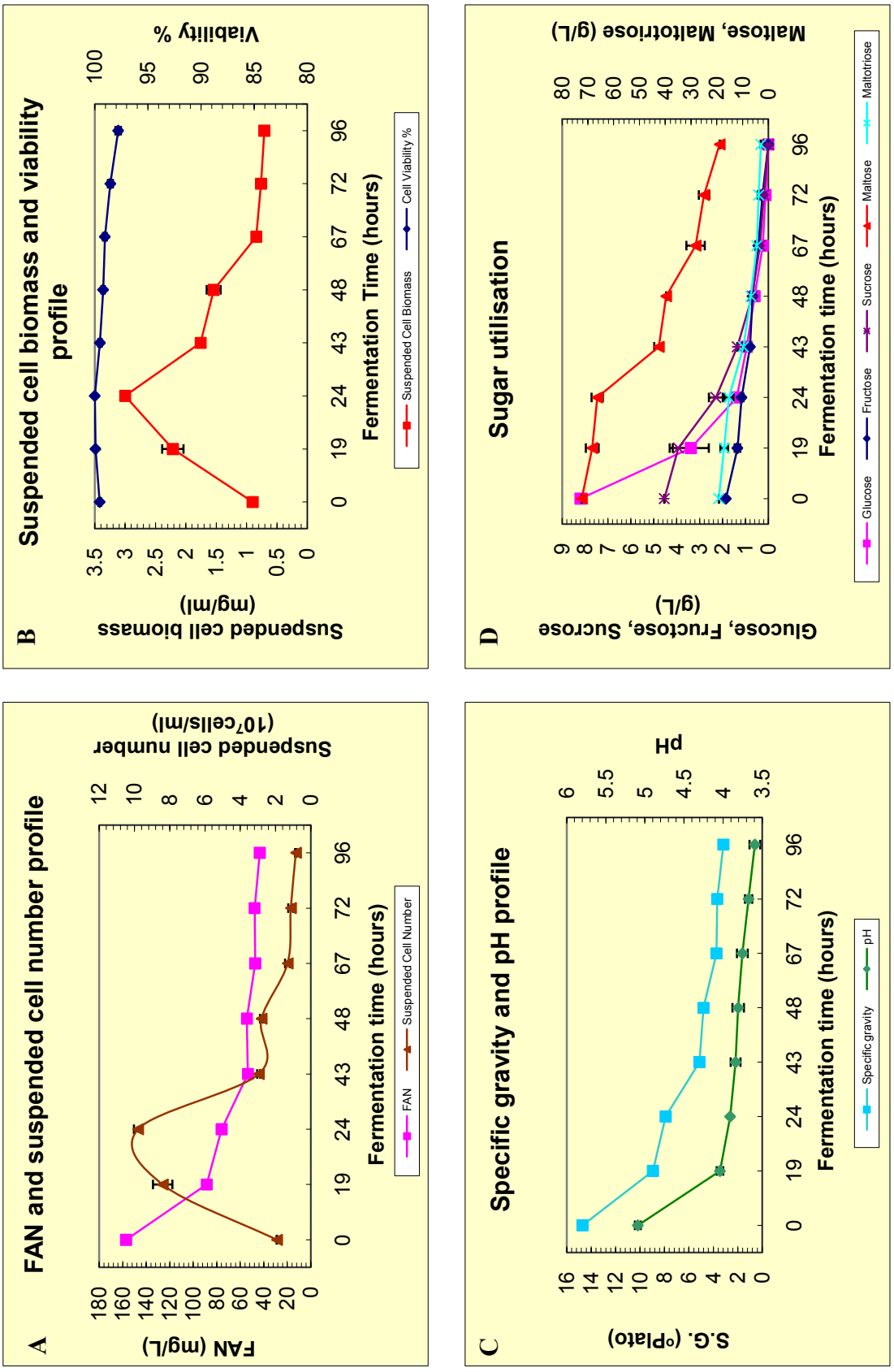


Figure 55: Fermentation profile and sugar utilization for the SC3 lager fermentations (control-unsupplemented experiments). The results are the mean values of three fermentations ± S.D.

3.3.1.2 Amino acid and ammonia utilization

Figure 56 depicts the utilization of assimilable nitrogen during the control static fermentations (no additional amino acids or ammonia) conducted with the lager yeast strain SC3.

Glutamine and aspartic acid were the first nitrogen sources to have undergone complete absorption within 19h fermentation. Twenty four hours after the removal of these two amino acids from the fermentation medium, complete utilization of the rest of the Group 1 amino acids followed (**Figure 56A**).

Examining of the consumption behaviour of the Group 2 amino acids, revealed that methionine, once again was removed by the yeast cells within the first 24h of yeast incubation (**Figure 56B**). Histidine was then utilized after 43h of yeast incubation. Thereafter, isoleucine and leucine were depleted from the medium 5h after the utilization of histidine. Valine was the last nitrogenous material of this category to be utilized; its concentration was exhausted 72h after pitching.

Tryptophan, tyrosine and phenylalanine were found to be entirely exhausted also after 48h of fermentation. Following that, alanine was found to have been depleted after 67h of incubation and 5h later, the glycine wort content was also completely removed from the medium.

Proline decreased gradually after 43h of fermentation until the end of the incubation period (**Figure 56D**). Ammonia wort concentration reduced progressively during the course of the fermentation and then after 72h it was found that ammonia levels started to be excreted again into the fermenting wort.

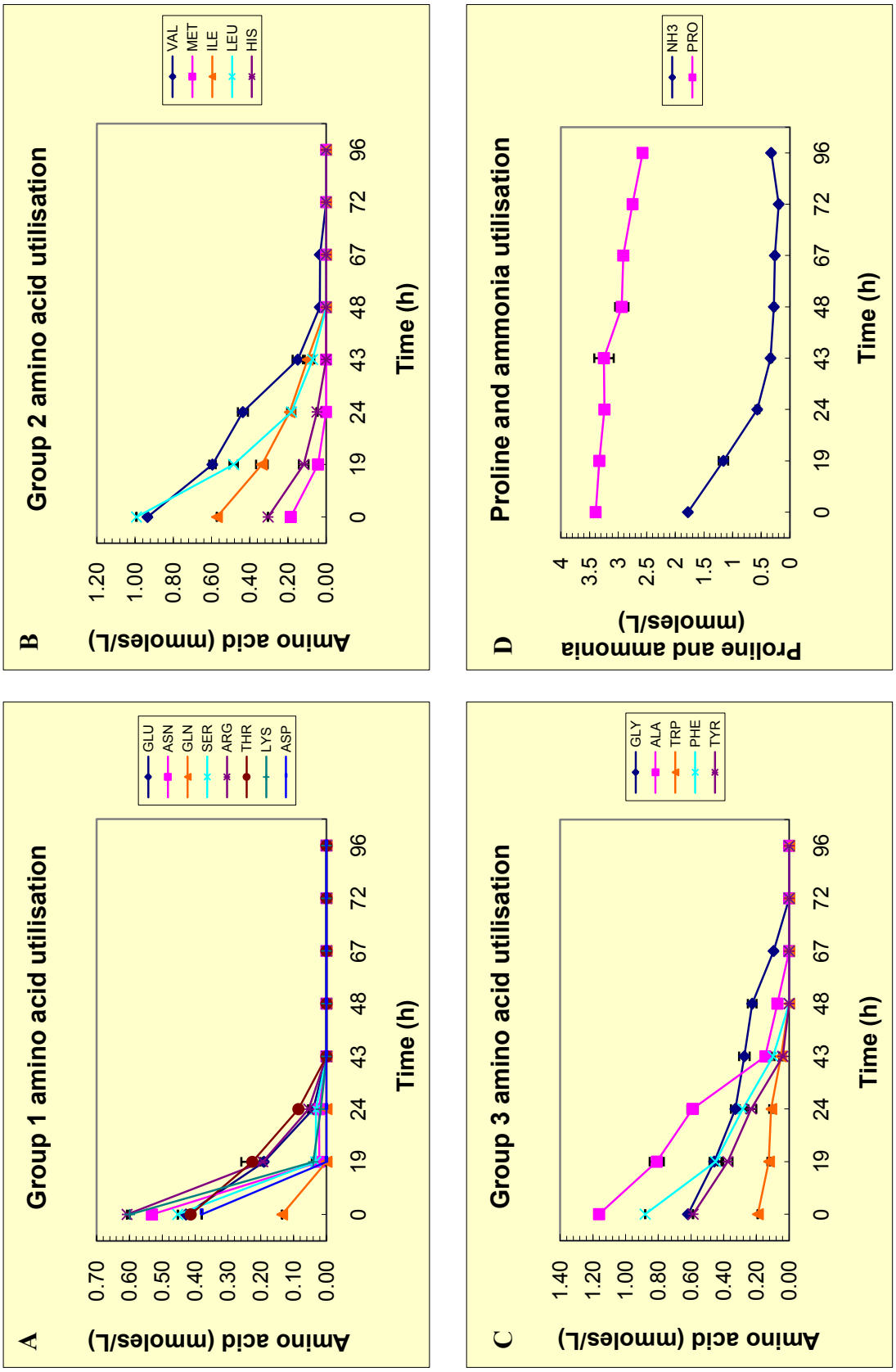


Figure 56: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the SC3 static control fermentations. The results are the mean values of three fermentations ± S.D.

3.3.1.3 Wort nitrogen distribution and nitrogen source utilization

Figure 57 illustrates a summary of detailed nitrogen uptake before and after fermentation in terms of the percentage of the total wort nitrogen each individual nitrogenous wort constituent takes up for the control static fermentations conducted with the lager yeast stain SC3. In addition, the last section of this figure shows the total percentage utilization for each nitrogenous wort compound (**Figure 57C**).

Amino acids that are components of Group 1, according to their absorption rate, were found to constitute approximately 3% of the total yeast utilizable nitrogen of the unfermented wort. Similarly, when mass balance calculations were carried out for Group 2 amino acids, it was found that the nitrogen portion that they constitute in the unfermented wort was 0.5% less than the percentage of Group 1 nitrogen sources. When the nitrogen distribution of Group 3 amino acids was calculated, it was found to comprise the same percentage as Group 1 amino acids. Finally, ammonia and proline were found to represent approximately 15% of the total wort nitrogen before yeast pitching (**Figure 57A**).

When this series of control fermentations were complete, it was found that all the amino acid nitrogen sources of the 3 groups did not represent any portion of the fermented wort nitrogen; consequently they have all been consumed during fermentation. Nevertheless, proline and ammonium ions were found together to constitute 13% of the fermented wort nitrogen concentration (**Figure 57B**). The remaining 77% of the nitrogen wort content is in all likelihood the sum of small wort peptides that also play a significant role in yeast nitrogen metabolism.

As it was already discussed, all the amino acids exhibited 100% utilization, except for proline, just 10%. With ammonia, only a negligible proportion of 3% of its initial wort levels was left unutilized at the end of fermentation.

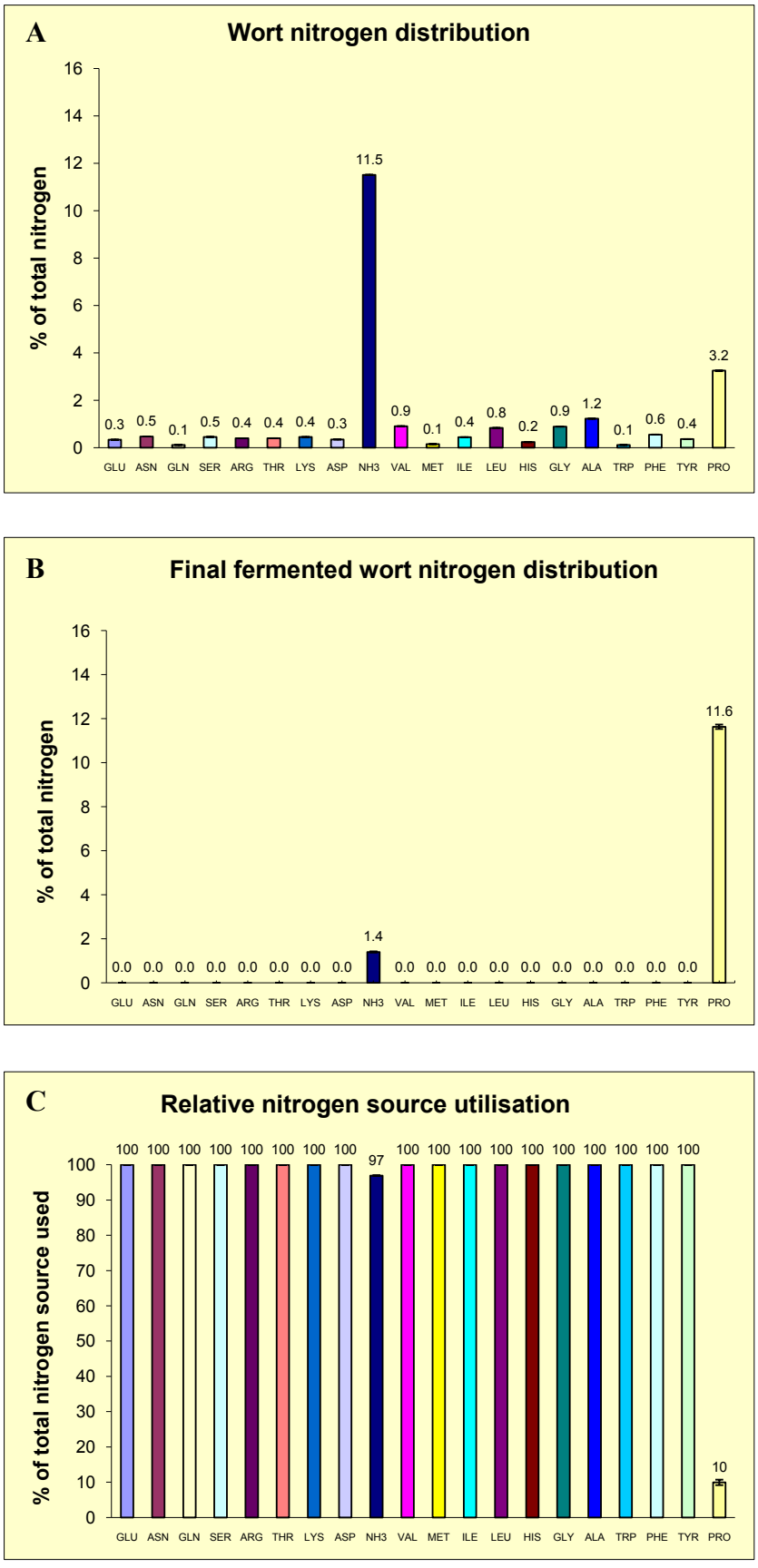


Figure 57: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (SC3 control lager static fermentations). The results are the mean values of three fermentations ± S.D.

3.3.1.4 Final fermentation measurements

Table 16: Final measurements for **SC3 control lager** fermentations (after 96h fermentation at 15°C and incubation at 4°C for 24h)

Parameter / Cylinder	ABV (%)	Total Wet Yeast Biomass (g)	Total Dry Yeast Biomass (g)	Viability (%)
Cylinder 1	6.612	30.953	22.548	97.72
Cylinder 2	6.318	31.562	23.934	97.64
Cylinder 3	6.231	31.738	23.683	98.47
Average ± S.D.	**6.387 ± 0.200**	**31.418 ± 0.412**	**23.388 ± 0.738**	**97.94 ± 0.458**

Table 16 depicts the final readings recorded for the sum of control static SC3 lager fermentations, which were conducted simultaneously with the SC3 supplemented fermentations. More specifically, the final measurements include the ABV%, the dry and wet biomass and the percentage of viable yeast cells.

The ABV% value was measured and it was found to be 6.39%, nearly the same as the first set of the SC3 lager fermentations (6.34%). Moving onto the next recorded reading, the average final yeast crop in wet form was found to be 31.4g produced from the 1.5l fermentation. The average dry yeast crop was 8g less (23.4g), after this yeast crop quantity was left to dry for a week. As for the total final yeast viability, it was found to be 98%.

3.3.2 Lysine supplemented fermentations (five times)

3.3.2.1 Fermentation profile and sugar utilization

Figure 58 illustrates the overall fermentation profile recorded during the static fermentations supplemented with five times the natural wort concentration of lysine in wort (15°Plato containing 30% VHM syrup). The yeast strain used for these fermentations was the lager strain SC3 and the temperature of the incubation room was 15°C.

The suspended yeast cell concentration peaked at $13x10^7$cells/ml, 19h into fermentation. FAN wort concentration declined very sharply for the first 19h of the experiment and then continued its reduction pattern for a further 24h when nitrogen levels remained constant for the last 5h of the experiment (**Figure 58A**).

Cell viability underwent almost no reduction, it being maintained at 99% after the completion of the fermentation. Cell biomass reached its maximum value, as with cell number, during the first 19h of incubation, which was 4.3mg/ml of sample and then it started decreasing gradually until the end of the experiment (**Figure 58B**).

Specific gravity decreased very rapidly during the fermentation, especially during the first stages of the experiment, which exhibited an 8°Plato reduction within the initial 19h of fermentation. The wort gravity continued to reduce steadily and it reached its target value in just 2 days of fermentation. The pH was found to have followed a similar decrease pattern as FAN and specific gravity, whose value reduced constantly for the first 43h of the incubation with the only difference that an insignificant increase was observed during the last 5h of the experiment (**Figure 58C**).

The fermentable sugars glucose, sucrose and fructose were all exhausted at the same time (48h), after gradually being taken up by the yeast cells. In addition, the maltose concentration underwent a significant reduction but without being completely absorbed. Finally, maltotriose also showed a significant reduction pattern during the whole experimental progress; however it was not taken up entirely (**Figure 58D**).

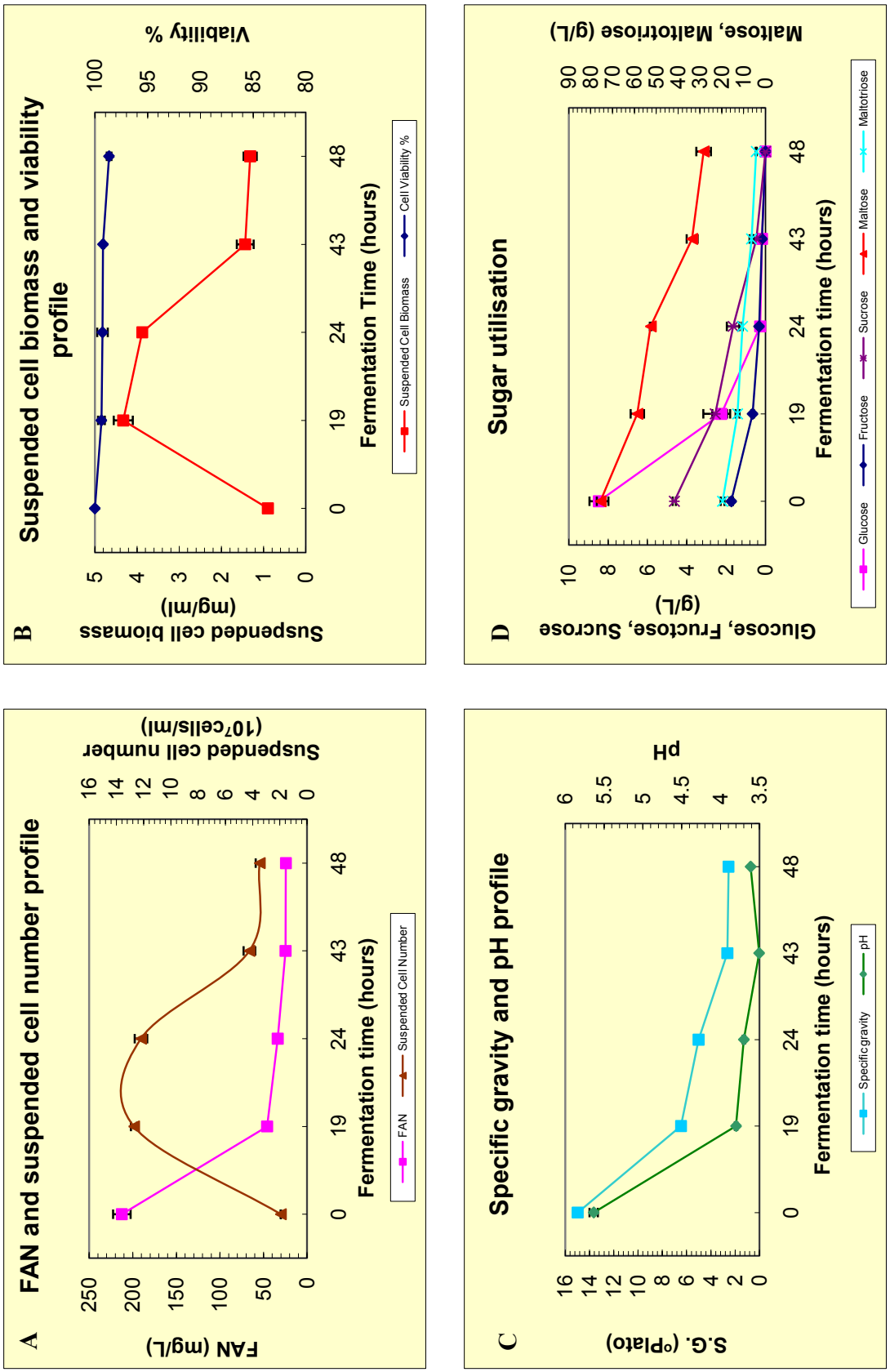


Figure 58: Fermentation profile and sugar utilization for the SC3 lager static fermentations (x5 Lysine supplementation). The results are the mean values of three fermentations ± S.D.

3.3.2.2 Amino acid and ammonia utilization

Figure 59 illustrates the uptake of amino acids and ammonium ions from the wort fermentations supplemented with five times the natural wort concentration of lysine (3mmol/l lysine). Again, the lager yeast strain SC3 was used as the pitching yeast. As it is shown in **Figure 59A**, lysine supplementation facilitated the absorption of the Group 1 amino acids, since all of them, excluding arginine were used rapidly and depleted within 19h after the start of the fermentation. Complete lysine uptake occurred after 48h fermentation and that was not expected since its initial wort level was five times more than the normal, which means more time was needed for complete lysine utilization.

Considering the second amino acid group, the same effect was also observed for the nitrogen sources methionine, leucine, isoleucine and histidine, which were consumed entirely by the dividing yeast cell during the first 19h of fermentation. Valine was the only amino acid in Group 2, which was depleted after 43h fermentation (**Figure 59B**).

When the Group 3 amino acids absorption rate was considered it was found that the majority of assimilable nitrogen was taken up after 24h fermentation, leaving only glycine, which was removed efficiently 24h after the utilization of the rest of the amino acids. The uptake of most Group 3 amino acids was very rapid, while the uptake of glycine occurred in phases (**Figure 59C**).

It would appear that the addition of lysine had also a stimulatory effect on the utilization of ammonia, which was removed from the fermentation medium after only 19h incubation. However, proline did not participate in the nitrogen metabolism of the yeast, as no uptake of this nitrogen source occurred (**Figure 59D**).

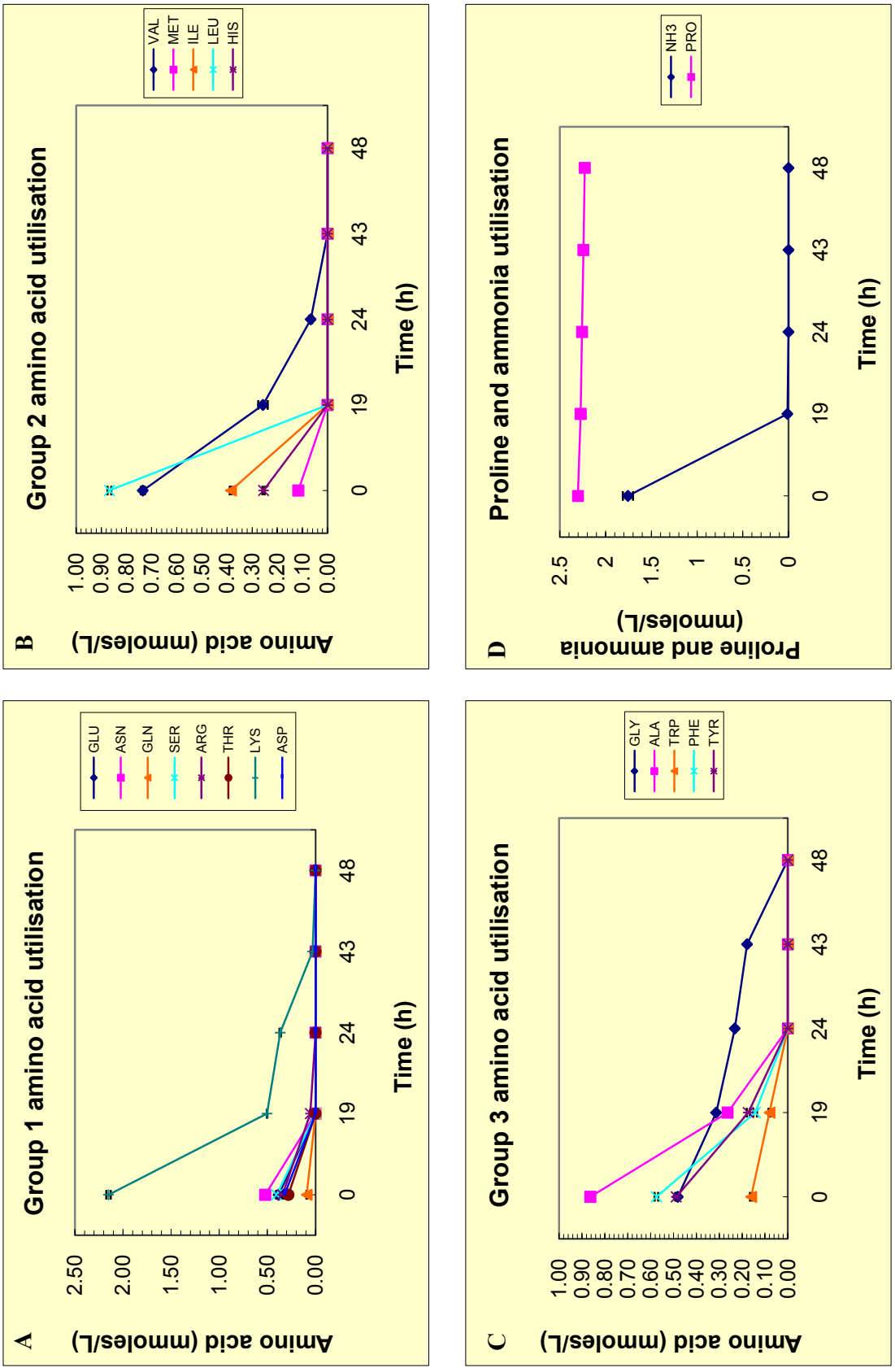


Figure 59: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the SC3 static lager fermentations (x5 Lysine supplementation). The results are the mean values of three fermentations ± S.D.

3.3.2.3 Wort nitrogen distribution and nitrogen source utilization

Figure 60 illustrates the detailed nitrogen distribution before and after fermentation in terms of the percentage of total wort nitrogen each individual amino acid and ammonia constitutes for the high gravity wort (15^{o}Plato + 30 VHM syrup) static fermentations supplemented with five times more the natural wort concentration of lysine. In addition, **Figure 60C** shows the percentage utilization of each wort nitrogen source.

Amino acids that exhibit very rapid absorption (Jones and Pierce, 1964) were found to constitute 4.5% of the total nitrogen in unfermented wort. Such an increase compared to the control fermentations was induced due to the use of additional lysine in the fermentation medium. Amino acids that undergo intermediate absorption were found to represent 3.2% of the total nitrogen in unfermented wort, whereas amino acids with slow absorption constitute 3.7% of the total wort nitrogen concentration. After completion of the lysine supplemented fermentations none of these free amino nitrogen constituents, regardless their utilization speed, was detected in the fermented wort. The same observation was also valid for ammonia, which was depleted at the end of these experiments. As expected, proline, which constitutes 3.2% of the total wort nitrogen, was not utilized efficiently with the result that 16% of the nitrogen content of the fermented wort was proline, while the rest of it, small peptides (**Figure 60B**). Ammonia was estimated to be almost 16% of the total nitrogen content in unfermented wort, while at the end of fermentation, no ammonia remained in the medium.

All the amino acids that represent yeast utilizable nitrogen content were depleted at the end of the experiment by having achieved 100% utilization, including ammonia. Such an observation was not valid for proline, because almost none (only 3%) of its original concentration was consumed.

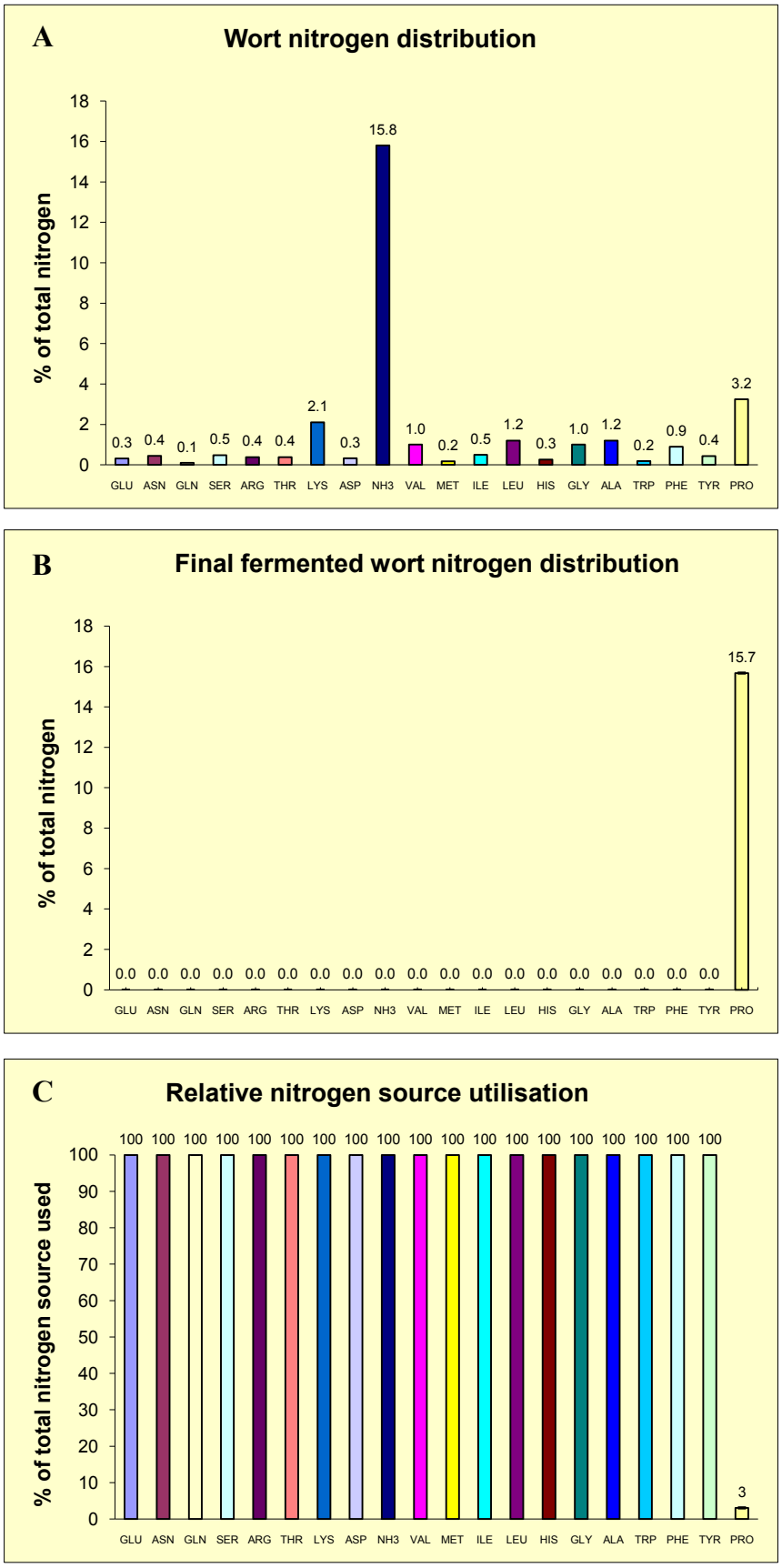


Figure 60: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (SC3 x5 Lysine supplemented lager static fermentations). The results are the mean values of three fermentations ± S.D.

3.3.2.4 Final fermentation measurements

Table 17: Final measurements for **x5 Lysine** fermentations
(after 48h fermentation at 15°C and incubation at 4°C for 24h)

Parameter / Cylinder	ABV (%)	Total Wet Yeast Biomass (g)	Total Dry Yeast Biomass (g)	Yeast Viability (%)
Cylinder 1	7.434	40.818	30.403	98.72
Cylinder 2	7.556	40.952	30.310	99.51
Cylinder 3	7.298	40.257	30.297	99.78
Average ± S.D.	**7.429 ± 0.129**	**40.676 ± 0.369**	**30.336 ± 0.058**	**99.34 ± 0.551**

The final experimental results of the lysine supplemented fermentations pitched with the lager strain SC3, are illustrated in **Table 17**. They show the total wet and dry yeast crop, viability and total alcohol production after the fermented wort achieved the desired halting gravity and twenty four hour maturation at 4°C.

The average ethanol percentage of the fermented wort was found to be 7.4% (v/v), which was the highest ABV% produced within all the fermentation trials that were carried out. This is another index confirming the fact that lysine wort supplementation facilitated the yeast metabolic activity and fermentation performance.

The average total wet yeast crop produced at the end of fermentation differs by nearly 10g from the total average dry biomass. Hence, these 10g appear to be fermented wort and trapped CO_2, which was evaporated after the oven treatment.

The final average yeast viability was measured and it was found to be similar (99.34%) to the viability of the cells at the beginning of fermentation.

3.3.3 Arginine supplemented fermentations (five times)

3.3.3.1 Fermentation profile and sugar utilization

Figure 61 illustrates the FAN, wort gravity and pH reductions and the yeast cell number, biomass and viability for the series of static fermentation in which arginine was used as a nitrogen wort supplement five times at its natural wort concentration. The pitching was again the lager strain SC3. The time taken for the fermentations to reach the desired final gravity was 67h.

Maximum number of yeast cells was obtained during the first day of fermentation ($13x10^7$cells/ml). During the next 24h incubation, cell number fell to less than half of its initial maximum value. FAN wort content started to be utilized efficiently for the first 43h of fermentation and then, FAN levels remained constant until the end of the incubation period (**Figure 61A**).

Cell viability after pitching increased by 1% reaching almost 100% of viable cells, which was retained for 24h and after that viability reduced to its initial value and remained constant until the completion of fermentation. Biomass increased rapidly reaching 4mg/ml of sample 24h after pitching, but at the end of fermentation, yeast in suspension had decreased to less than 1mg/ml of sample.

Specific gravity decreased significantly for the first 43h of the experiment and then noted a further slight reduction rate during the last 24h of the incubation. The pH exhibited a similar reduction pattern as FAN, declining gradually for 43h and then remaining at almost the same level until the end of fermentation (**Figure 61C**).

Complete uptake of the reducing sugars sucrose, fructose and glucose was achieved after 67h yeast incubation (**Figure 61C**). Furthermore, maltotriose also followed a similar continuous consumption rate but without being totally utilized. Maltose wort levels dropped sharply during the whole course of the fermentation. Nevertheless, this particular fermentable sugar was not totally utilized.

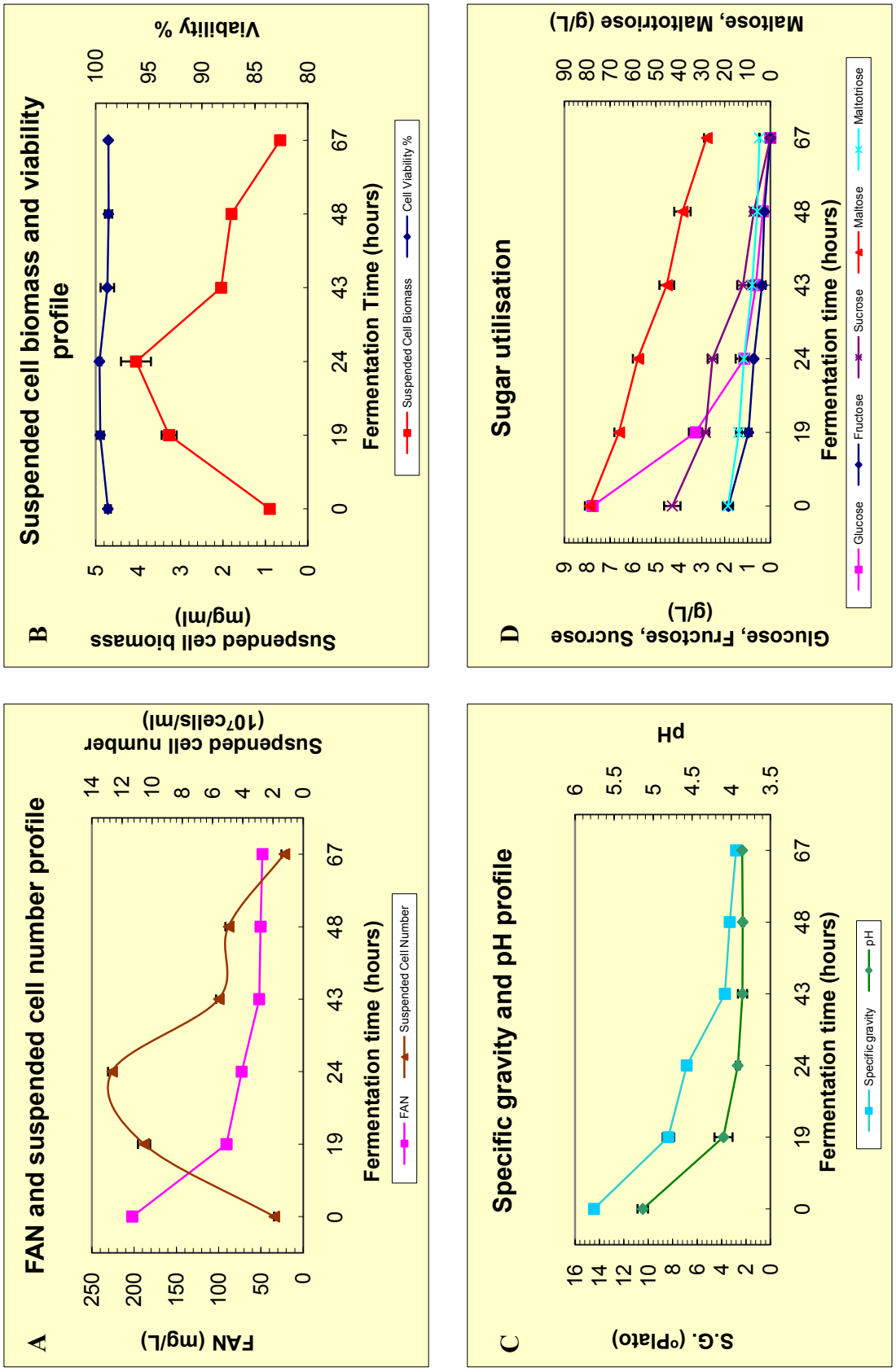


Figure 61: Fermentation profile and sugar utilization for the SC3 lager static fermentations (x5 Arginine supplementation). The results are the mean values of three fermentations ± S.D.

3.3.3.2 Amino acid and ammonia utilization

Figure 62 illustrates the uptake of amino acids and ammonia during the SC3 lager fermentations in which arginine was used as a nitrogen supplement, five times its initial wort concentration levels (3mmol/L).

Figure 62A presents the amino acids that underwent a rapid uptake. As it can be seen, the amino acids of this group that were removed immediately, within 19h, were lysine, glutamate, glutamine, asparagine, aspartate and serine. Then, complete utilization of threonine and arginine followed, which were fully removed from the wort after 43h and 67h of fermentation, respectively.

In Group 2 amino acids, methionine was also an amino acid that could not be detected after 19h fermentation (**Figure 62B**). Then, isoleucine and histidine were taken into the yeast cytosol after 24h of fermentation followed by leucine, which was completely assimilated during the next 19h. Valine was found to be the last amino acid of the second group to be utilized by the yeast, 19h before the completion of the experiment.

Tryptophan and phenylalanine were the two Group 3 amino acids, which were utilized completely within the first 43h of fermentation (**Figure 62C**). Tyrosine and alanine were exhausted from the medium 5h following complete utilization of tryptophan and phenylalanine. Finally, glycine was consumed gradually during fermentation, but without being exhausted at the end.

Ammonia wort levels declined gradually and after 48h fermentation were depleted. Proline exhibited a typical absorption pattern for proline without being used by the yeast until the end of the incubation period.

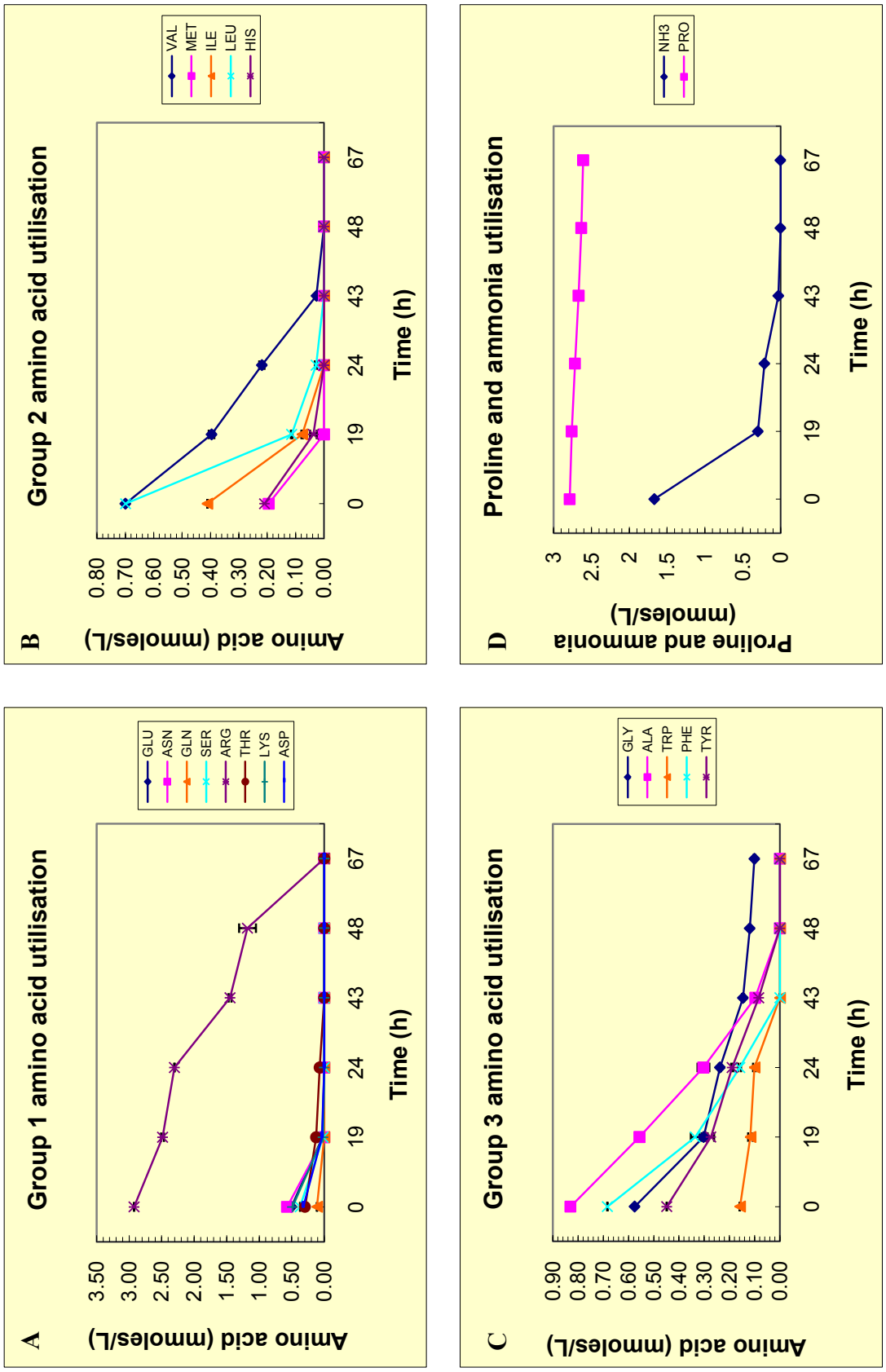


Figure 62: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the SC3 lager static fermentations (x5 Arginine supplementation). The results are the mean values of three fermentations ± S.D.

3.3.3.3 Wort nitrogen distribution and nitrogen source utilization

Figure 63 illustrates the nitrogen wort distribution before and after fermentation. It explains what quantity of each individual amino acid and ammonia exists in unfermented and fermented wort with respect to the total nitrogen content. This nitrogen configuration relates to high gravity wort static fermentations supplemented with five times more than the natural wort concentration of arginine. In addition, **Figure 63**C depicts the percentage utilization of each wort nitrogenous compound.

Amino acids classified in Group 1 constitute 3.4% of the total nitrogen in unfermented wort and such an increase in their fraction was an effect of arginine supplementation in the fermentation medium. Amino acids that undergo intermediate absorption were found to represent 1.6% of the total nitrogen in unfermented wort, whereas amino acids that belong in Group 3 constitute 2.3% of the total wort nitrogen concentration. Ammonia and proline, the only amino acid of Group 4, together represent nearly 12% of the initial wort nitrogen. The remaining nitrogen fraction, which is the nearly 81% of the total wort nitrogen, is believed to be nitrogenous combinations with two and three amino acid residues. On the other hand, none of the Group 1 and 2 amino acids were found to take part in the nitrogen distribution of the final fermented wort, including ammonia. However, the Group 3 amino acid glycine was found to constitute 0.6% of the total nitrogen of fermented wort. The other nitrogenous compound detected was proline, which constitutes 10% of the nitrogen content at the end of the fermentation. Thus, the sum of wort amino acids that were involved effectively in the nitrogen yeast metabolism, were found to have been completely utilized. This observation was confirmed for the ammonia wort levels, which was exhausted at the end of the incubation period. Again, only 6% of the total proline wort concentration was utilized by yeast cells (**Figure 63C**).

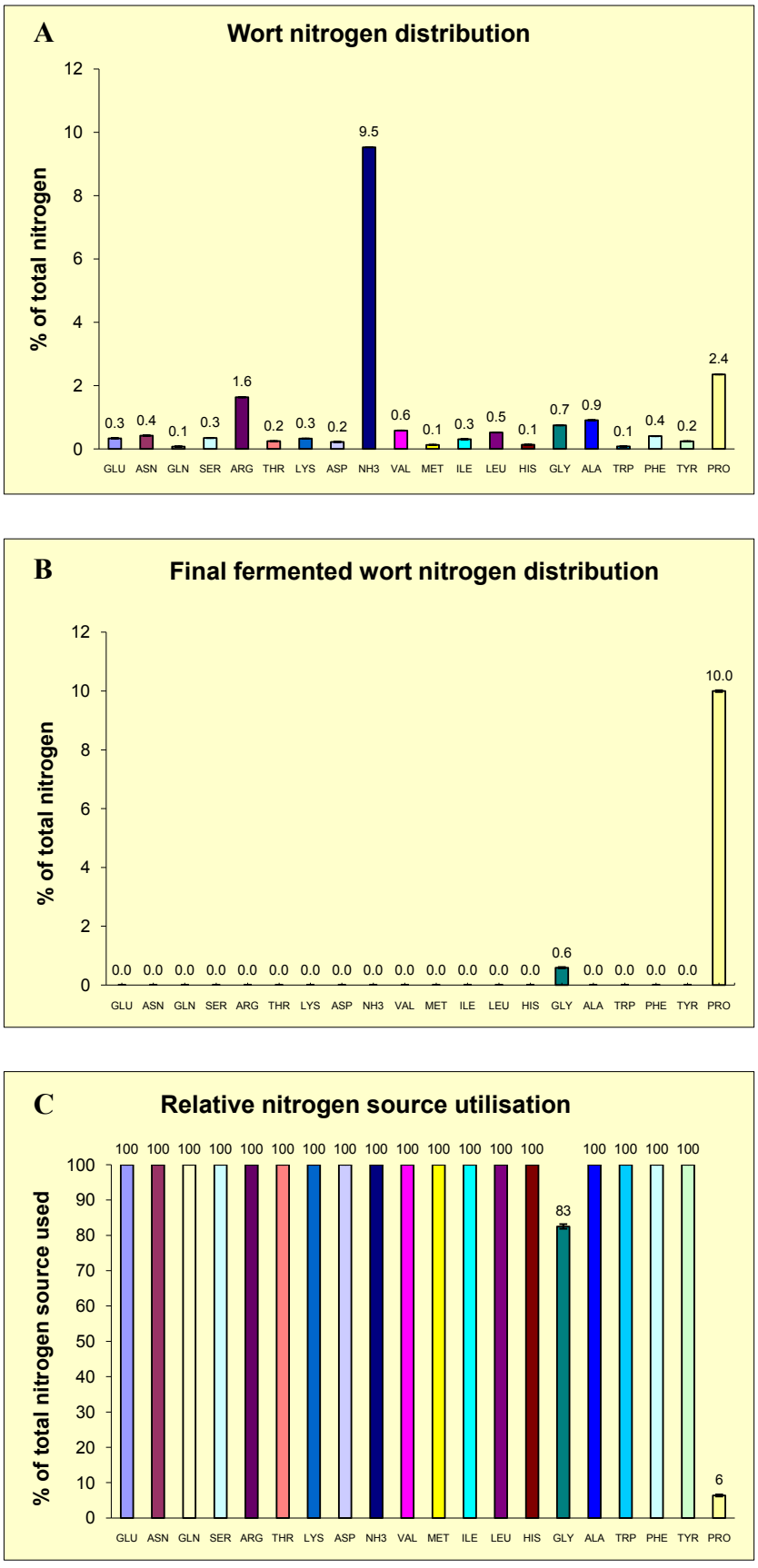


Figure 63: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (SC3 x5 Arginine supplemented lager static fermentations). The results are the mean values of three fermentations ± S.D.

3.3.3.4 Final fermentation measurements

Table 18: Final measurements for **x5 Arginine** fermentations (after 67h fermentation at 15°C and incubation at 4°C for 24h)

Parameter / Cylinder	ABV (%)	Total Wet Yeast Biomass (g)	Total Dry Yeast Biomass (g)	Viability (%)
Cylinder 1	6.640	35.082	25.600	99.065
Cylinder 2	6.882	36.156	26.480	99.512
Cylinder 3	6.909	36.295	26.553	99.383
Average ± S.D.	**6.810 ± 0.148**	**35.844 ± 0.664**	**26.211 ± 0.530**	**99.320 ± 0.230**

The final fermentation percentages for ABV%, total wet and dry yeast crop and cell viability for the supplemented fermentations with five times arginine than its basal wort concentration are shown in **Table 18**.

The average total alcohol composition was 6.8% (v/v), with no significant variations between the three different fermentation cylinders operating at the same time.

The total wet yeast biomass was found to be 35.8g and the dry version of yeast weight was 9.6g less.

The final average cell viability was 99.3%, indicating that almost all the yeast cells were viable after the completion of the fermentation and after 24h maturation.

Finally, it is worth mentioning that even though the arginine supplemented fermentations were conducted nearly 30h faster than the control fermentations, the final results illustrated in **Table 18** show that arginine supplementation did not generate any significant difference in terms of alcohol production and cell viability.

3.3.4 Methionine supplemented fermentations (five times)

3.3.4.1 Fermentation profile and sugar utilization

Figure 64 illustrates the progress of the fermentation factors during static fermentations in which methionine at five times its natural wort concentration was added in order to enhance the wort nitrogen content. The yeast used was again the lager strain SC3. Complete attenuation occurred after 103h fermentation.

Maximum cell number was obtained during the first day of fermentation ($7.5x10^7$cells/ml). Thereafter, cell number showed a continuous decline. FAN started decreasing rapidly for the first 43h of fermentation and then it continued to be at a constant level until the end of the experiment (**Figure 64A**).

Cell viability remained high throughout the experiment, decreasing only by 1% after 24h of fermentation and retaining this value until the end of fermentation. Biomass appeared to have reached its greatest value (3mg/ml of sample) 24h after pitching, where at the end of fermentation a 2.8mg/ml decrease was observed (**Figure 64B**).

Specific gravity decreased sharply for the first 43h of incubation and then continued at a slower reduction rate until its target value was reached. The pH declined sharply for 67h and then it continued to increase until the end of fermentation (**Figure 64B**).

A continuous concentration reduction was observed for all the wort fermentable sugars. In more detail (**Figure 64D**), glucose, sucrose and fructose were all utilized by the end of fermentation, after 103h. Maltose declined also during the fermentation having noted a 60g/L reduction at the end of the incubation period. Finally, maltotriose exhibited a linear utilization rate with very low residual levels left unconsumed after 103h fermentation.

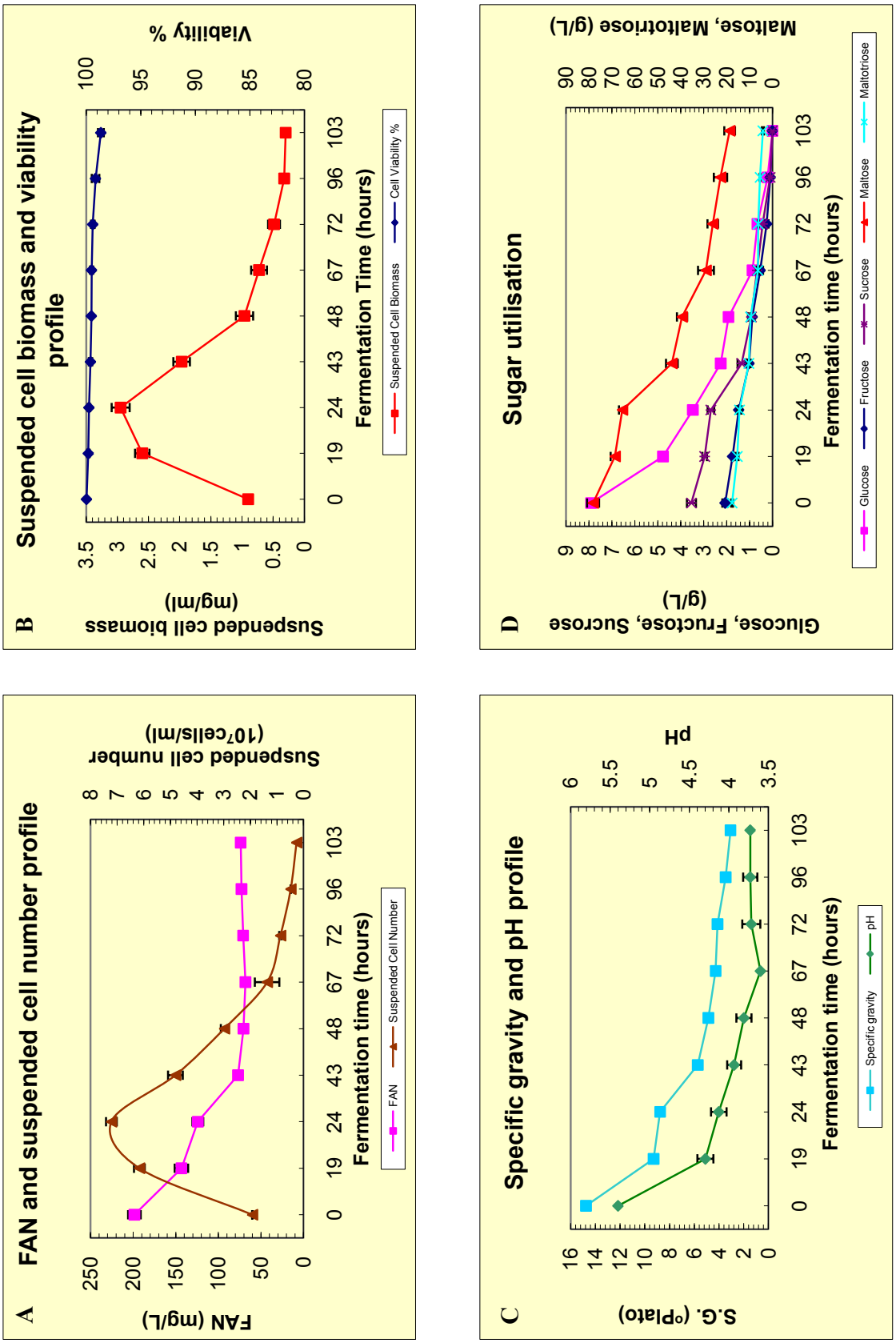


Figure 64: Fermentation profile and sugar utilization for the SC3 lager static fermentations (x5 Methionine supplementation). The results are the mean values of three fermentations ± S.D.

3.3.4.2 Amino acid and ammonia utilization

The general nitrogen uptake pattern for the SC3 lager yeast fermentations was enhanced with five times the natural wort concentration of methionine, is presented in **Figure 65**. The results obtained during the fermentations supplemented with two times methionine are not shown here since the similar effect was induced with the fermentations supplemented five times.

By examining **Figure 65A**, it can be seen that the time taken for the Group 1 amino acids to be exhausted was prolonged compared to the control (**Figure 56A**) and the other amino acid supplemented fermentations (**Figures 59A and 62A**). In other words, the majority of the amino acids were fully utilized by the yeast in 43h rather than sooner, usually during the first 24h of fermentation. In addition, the amino acids, arginine and threonine underwent complete absorption, after 67h and 72h of fermentation, respectively.

Methionine from Group 2 was the only amino acid of this group, which was removed from the wort within 48h of fermentation. Isoleucine and histidine were depleted after 67h of fermentation and finally valine and leucine were completely absorbed by the yeast 7h before the end of the experiment, after 96h (**Figure 65B**).

A similar consumption fate of leucine and valine was also observed for the Group 3 amino acids tryptophan, tyrosine, alanine and phenylalanine. Glycine was taken up at a constant rate during fermentation; however significant concentrations of this amino acid remained in the wort at the end of fermentation concluding that methionine excess in wort had an inhibitory effect on the complete utilization of glycine, probably due to the nitrogen catabolite repression phenomenon (**Figure 65C**).

Ammonia wort levels declined gradually until the end of yeast incubation, but without being totally removed from the wort. The proline concentration, even after the exhaustion of Group 1, 2 and 3 amino acids, remained unchanged indicating that yeast this time did not have any preference for this amino acid (**Figure 65D**).

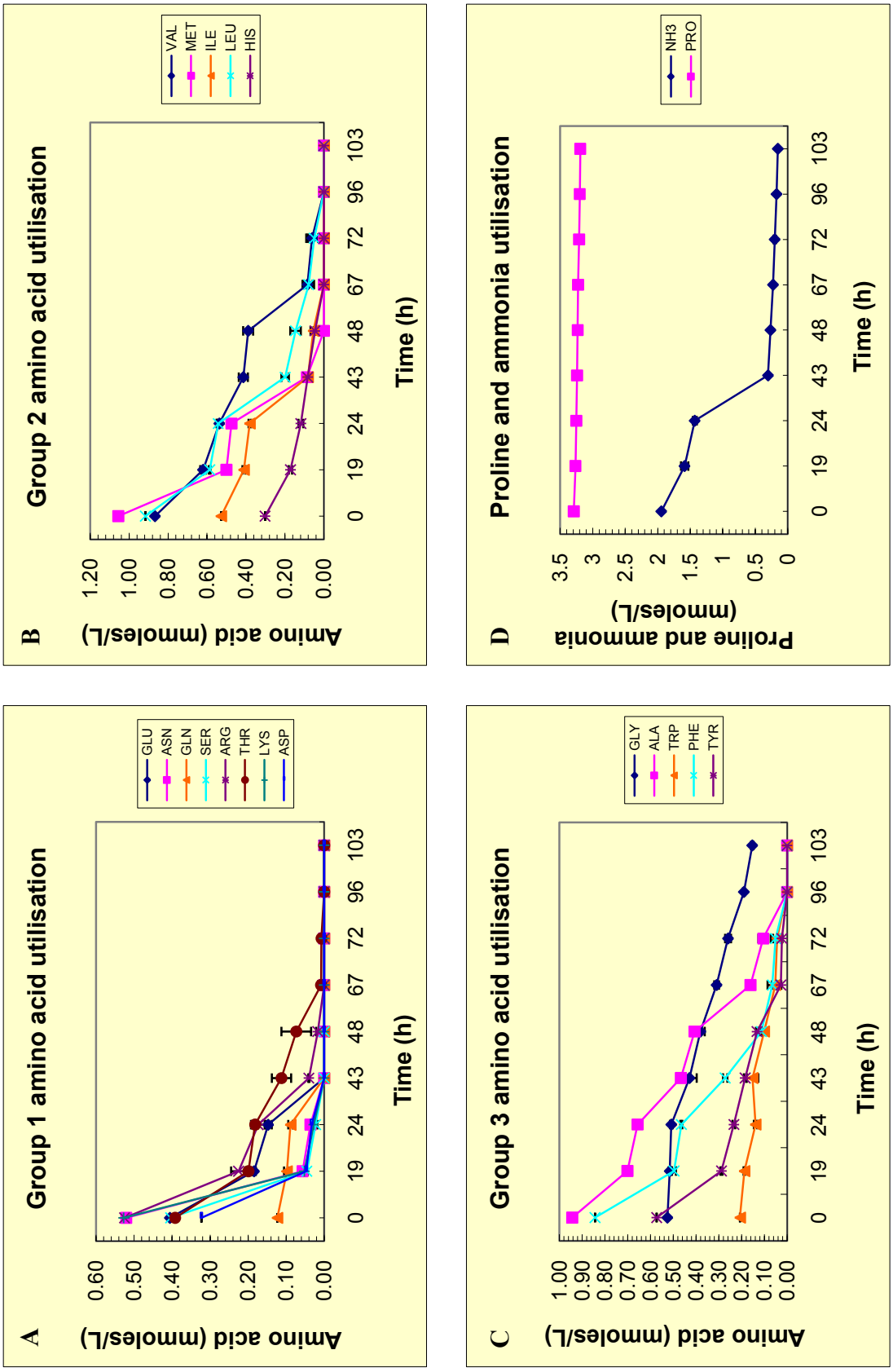


Figure 65: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the SC3 static lager fermentations (x5 Methionine supplementation). The results are the mean values of three fermentations ± S.D.

3.3.4.3 Wort nitrogen distribution and nitrogen source utilization

Figure 66 summarises the nitrogen allocation before and after fermentation in terms of the percentage of the total wort nitrogen each individual amino acids and ammonia for the high gravity static fermentations supplemented with five times more the natural wort concentration of methionine. In addition, **Figure 66C** depicts the percentage utilization of each wort nitrogen source.

Amino acids that exhibited very rapid absorption were found to constitute 3% of the total nitrogen in unfermented wort. Amino acids that undergo intermediate absorption were found to represent 3.6% of the total nitrogen in unfermented wort, where amino acids that are absorbed after the depletion of Groups 1 and 2 constitute 3.5% of the total wort nitrogen concentration. When the fermentation target gravity was achieved, only ammonia, proline and, this time, glycine remained unabsorbed (**Figure 66B**). In addition, it was estimated that they constitute 22.5% of the final nitrogen wort content and as it has been found in previous fermentations, the rest of the nitrogen proportion is assumed to be unused peptides.

As discussed above, all the amino acids that serve as nitrogenous yeast nutrients were depleted at the end of the experiment by having accomplished 100% utilization, excluding glycine and proline. To be more specific, 71% of the initial glycine wort concentration was utilized by the yeast and just 3% of proline. As for ammonia, its percentage utilization was found to be 92% of that available.

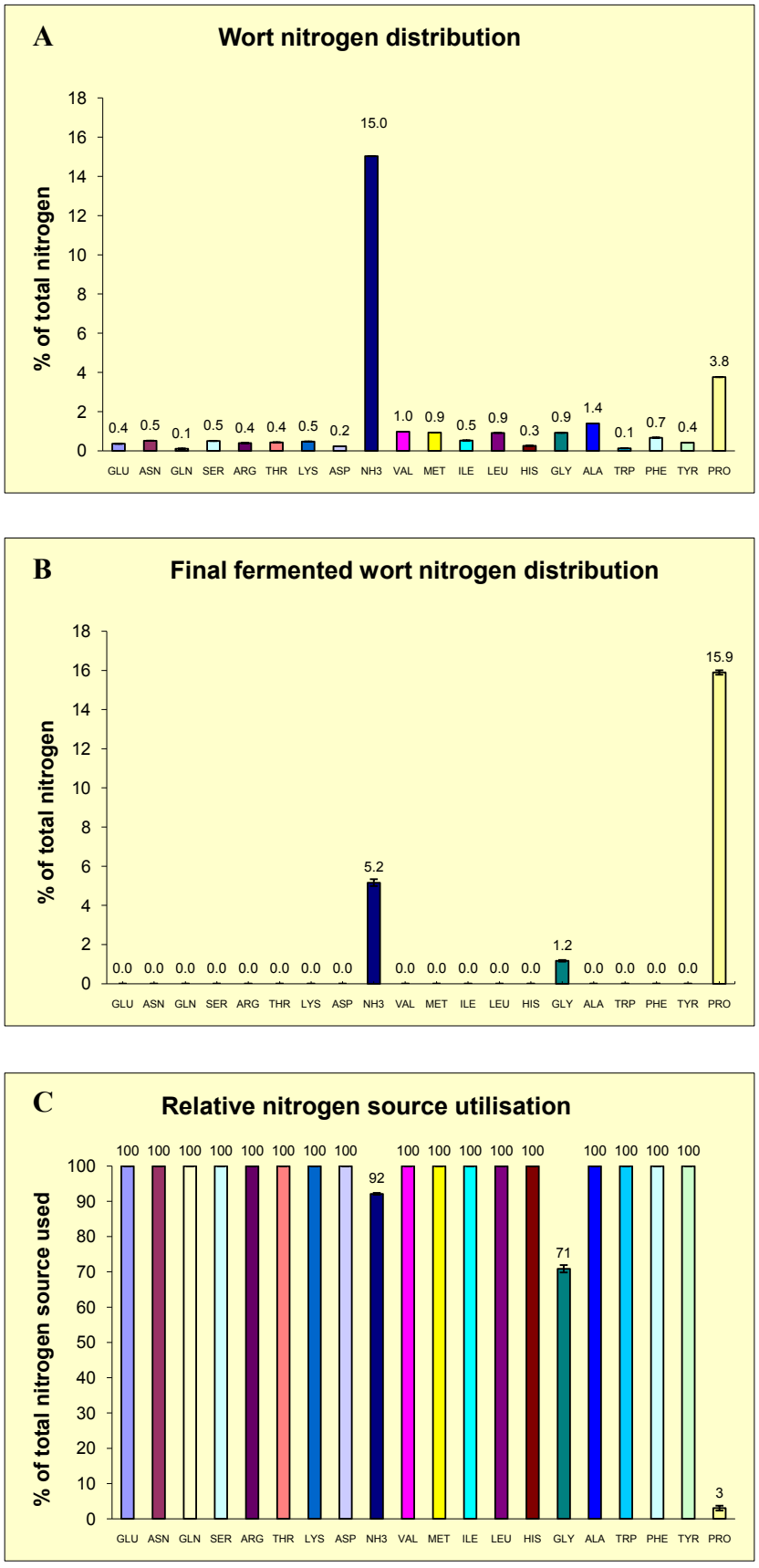


Figure 66: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (SC3 x5 Methionine supplemented lager static fermentations). The results are the mean values of three fermentations ± S.D.

3.3.4.4 Final fermentation measurements

Table 19: Final measurements for **x5 Methionine** fermentations (after 103h fermentation at 15°C and incubation at 4°C for 24h)

Parameter / Cylinder	ABV (%)	Total Wet Yeast Biomass (g)	Total Dry Yeast Biomass (g)	Viability (%)
Cylinder 1	6.847	30.870	20.040	99.28
Cylinder 2	6.094	30.140	20.344	100
Cylinder 3	6.370	30.310	20.400	98.92
Average ± S.D.	**6.437 ± 0.381**	**30.440 ± 0.382**	**20.261 ± 0.194**	**99.40 ± 0.550**

Table 19 depicts the final readings recorded for the static SC3 lager fermentations supplemented with five times more methionine than its original wort concentration.

The final measurements at the completion of fermentation include total ethanol production, dry and wet biomass and the percentage of viable yeast cells. The ABV% value from all the cylinders was found to be 6.44%, almost the same ethanol percentage produced at the end of the unsupplemented lager fermentations (6.39%). Such an effect shows us that methionine addition did not encourage higher ethanol production.

Both the total wet and dry biomass did not differ with the yeast crop collected at the end of the control fermentation illustrating again that methionine did not induce enhanced yeast cell proliferation. Similarly, a similar cell viability percentage was observed for both the methionine supplemented fermentations and the control.

3.3.5 Ammonia supplemented fermentations (twice)

3.3.5.1 Fermentation profile and sugar utilization

Figure 67 illustrates the overall fermentation profile recorded during the static fermentations supplemented with twice the natural wort concentration of ammonia. The yeast strain used for the conduct of these fermentations was again the lager strain SC3.

The suspended yeast cell concentration peaked at $9.5x10^7$cells/ml 19h into the fermentation. Thereafter, cells in suspension began to sediment, being still present in wort but having flocculate at the bottom of the fermentation vessels. That means that their number in suspension was reduced by $8x10^7$cells/ml during the next 24h. FAN wort content declined very rapidly for the first 43h of yeast incubation and then it exhibited a trivial increase for the next 24h. In continuance, FAN levels started to decrease until 163h of fermentation and at the end an insignificant augmentation in its value was also observed (**Figure 67**A).

The viability of cells in suspension was satisfactory throughout the whole experiment at 98%. Cell biomass reached its maximum level during the first 19h of incubation, which was 3.1mg/ml of sample and then it started decreasing gradually until the end of the experiment.

Specific gravity decreased continuously but very sluggishly throughout the fermentation, it reduced less than one ^{o}Plato every day, after 43h of incubation. The pH was found to decrease for the first 43h of fermentation, then more or less remained constant for 53h and finally it began to increase until the experiment was completed.

The fermentable sugars sucrose and fructose were both completely taken up after 115h incubation followed by glucose, whose uptake was complete after 163h fermentation. As for maltose and maltotriose, significant amounts of these sugars remained unabsorbed in the fermented wort (**Figure 67**D).

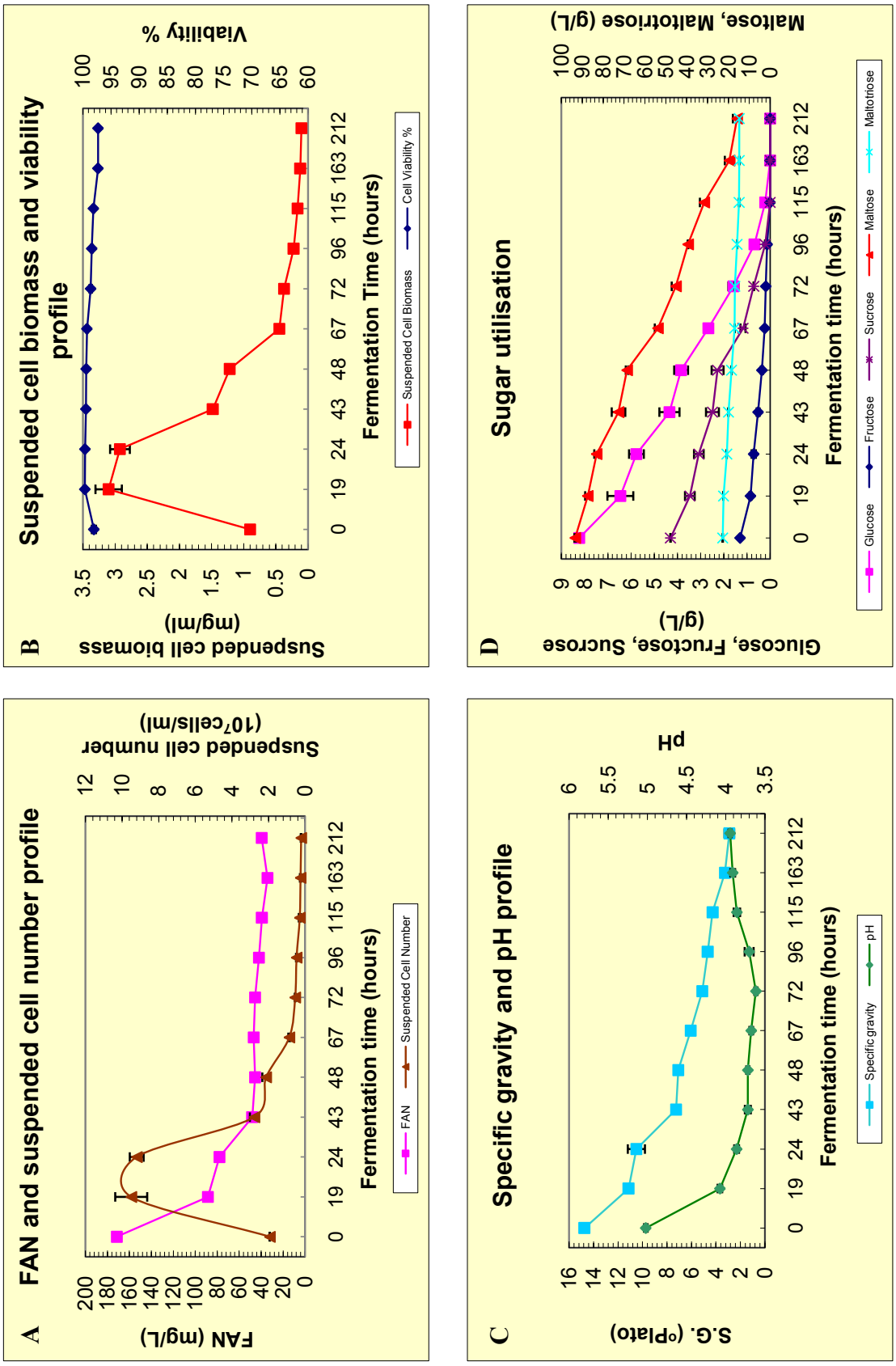


Figure 67: Fermentation profile and sugar utilization for the SC3 lager static fermentations (x2 NH_3 supplementation). The results are the mean values of three fermentations ± S.D.

3.3.5.2 Amino acid and ammonia utilization

Figure 68 illustrates the general nitrogen uptake for the SC3 lager yeast fermentations in which the wort type used was supplemented with twice the natural wort concentration of ammonium ions. By examining **Figure 68A**, the time taken for the Group 1 amino acids serine, lysine, arginine, threonine, aspartic acid and asparagine to be exhausted was just 24h. The remains of the nitrogenous yeast nutrients belonging to this category (glutamine and glutamic acid) were completely after 19h and 43h of fermentation, respectively (**Figure 68A**).

Methionine was also the only amino acid in Group 2, which disappeared from the wort within 24h fermentation. After 43h, isoleucine was depleted and finally 5h later valine, leucine and histidine were also completely removed from the fermenting wort (**Figure 68B**).

Tryptophan in Group 3 was also observed to have simultaneously undergone complete utilization with isoleucine, after 43h of fermentation. Tyrosine and phenylalanine were fully been taken up after 48h incubation. Surprisingly, alanine, while it started to be consumed very effectively for the first 48h of fermentation, plateaued for the rest of the incubation period. A similar effect was also observed for glycine, which was used efficiently for the first 48h of the experiment, however significant amounts of this amino acid remained unabsorbed until fermentation completion indicating that ammonia supplementation had an inhibitory effect on the assimilation of these two amino acids of the 3rd Group (**Figure 68C**). Ammonia utilization followed a similar absorption pattern to glycine, where ammonia concentration declined gradually for 96h of fermentation and then surprisingly was totally removed from the fermentation medium within the next 19h of incubation. Proline, as expected from other experiments, remained almost unchanged throughout the 212h of fermentation indicating that yeast did not have any preference for this particular amino acid due to lack of oxygen, after the enrichment of wort with extra ammonia (**Figure 68D**).

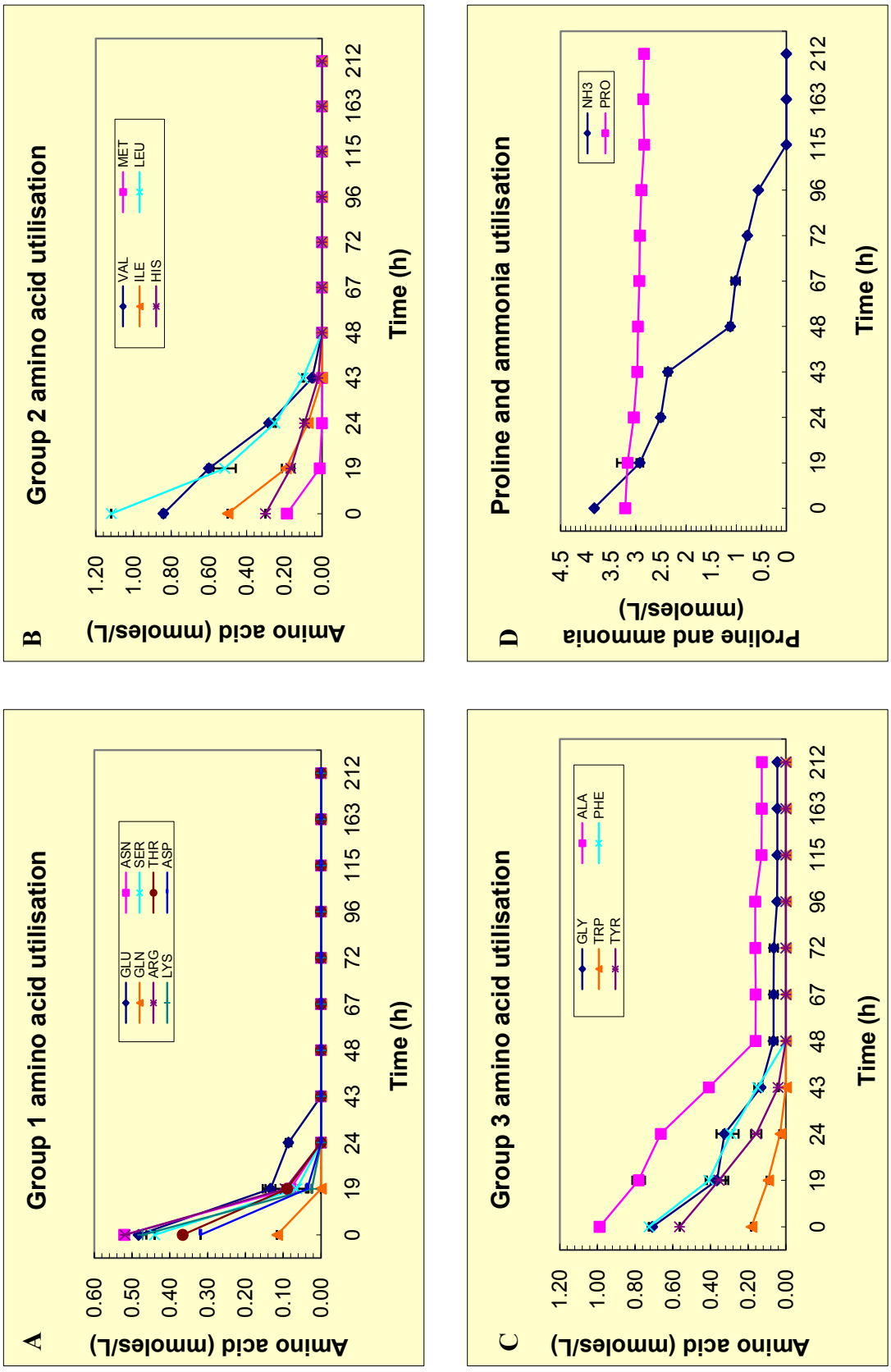


Figure 68: Amino acid and ammonia uptake according to the Jones and Pierce (1964) grouping for the SC3 static lager fermentations (x2 NH_3 supplementation). The results are the mean values of three fermentations ± S.D.

3.3.5.3 Wort nitrogen distribution and nitrogen source utilization

Figure 69 shows the nitrogen wort distribution before and after fermentation and the nitrogen utilization at the end of the ammonia supplemented fermentations.

As it can be seen from **Figure 69A**, amino acids that have been classified in Group 1 constitute 2.8% of the total nitrogen in unfermented wort. Amino acids that belong to Group 2 were found to represent 1.6% of the total nitrogen in unfermented wort, whereas amino acids of the 3rd Group constitute 3.2% of the total wort nitrogen concentration. Ammonia and proline together represent 25.5% of the initial wort nitrogen, since ammonium ions were used in order to enrich the nitrogenous material spectrum of the fermentation medium.

After fermentation completion (**Figure 69B**), none of the Group 1 and 2 amino acids were detected in the nitrogen distribution of the fermented wort, including ammonia. However, Group 3 amino acids glycine and alanine were found to constitute 0.24 and 0.48% of the total nitrogen of the fermented wort, respectively. The other nitrogenous material detected was proline, which constitutes 10% of the nitrogen content at the end of the fermentation.

The extent of utilization of the remaining nitrogenous materials in the fermented wort is 86% for glycine and 88% for alanine. As for the rest of the amino acids and ammonium ions, regardless of their group origin, they exhibited 100% utilization, except from proline, where only 10% of its initial wort concentration was successfully assimilated by the suspended yeast cells (**Figure 69C**).

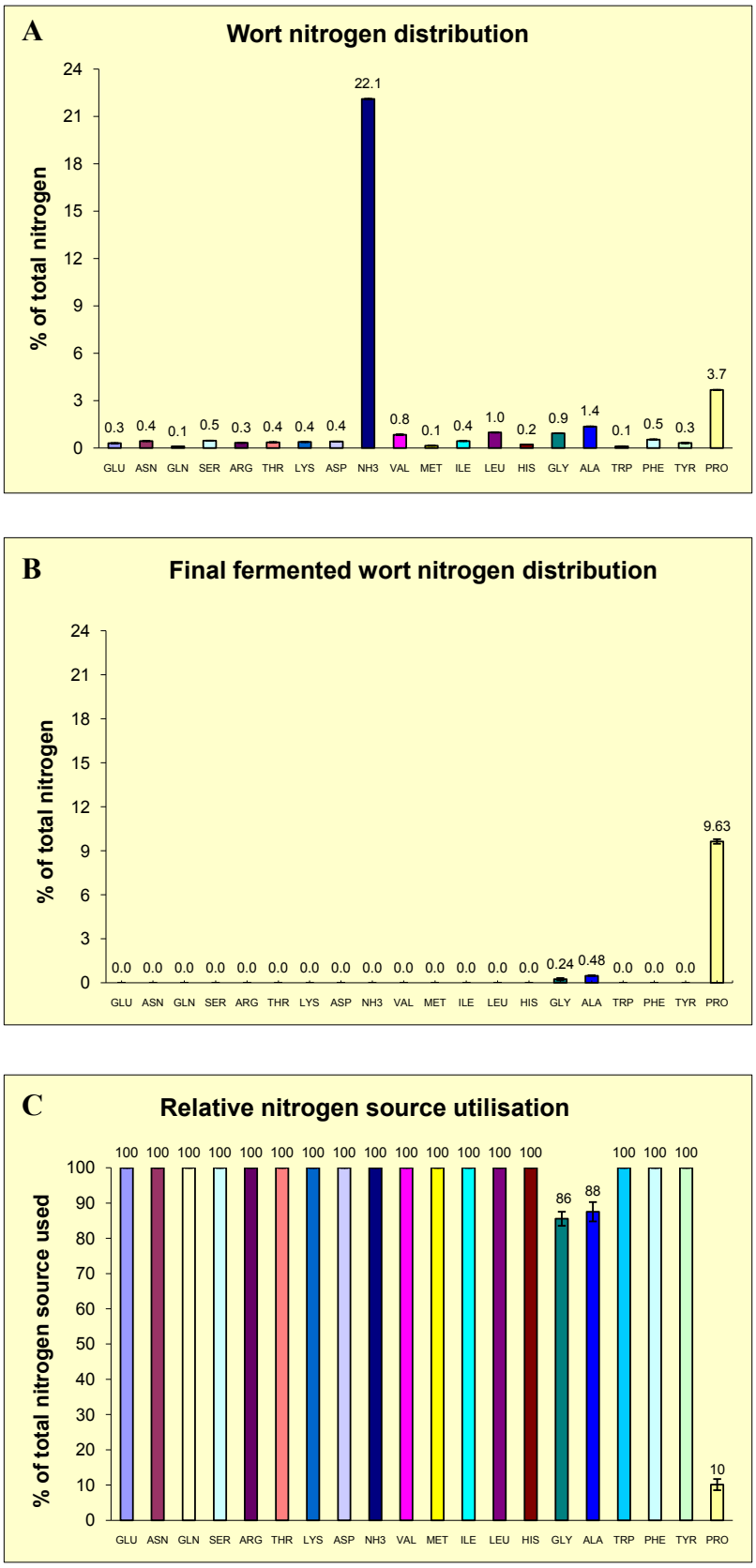


Figure 69: Total nitrogen wort distribution before and after fermentation and relative nitrogen utilization source (SC3 x2 NH_3 supplemented lager static fermentations). The results are the mean values of three fermentations ± S.D.

3.3.5.4 Final fermentation measurements

Table 20: Final measurements for **x2 NH_3 supplemented** fermentations (after 212h fermentation at 15°C and incubation at 4°C for 24h)

Parameter / Cylinder	ABV (%)	Total Wet Yeast Biomass (g)	Total Dry Yeast Biomass (g)	Viability (%)
Cylinder 1	6.604	27.731	20.292	90.035
Cylinder 2	6.766	31.618	23.614	89.275
Cylinder 3	6.827	33.309	25.754	91.67
Average ± S.D.	**6.732 ± 0.115**	**30.886 ± 2.860**	**23.220 ± 2.752**	**90.33 ± 1.224**

Table 20 summarizes the final results recorded for the sum of the static SC3 lager fermentations supplemented with twice the ammonia than its original wort concentration.

The final measurements after the completed fermentation include the total ethanol production, the dry and wet biomass and the percentage of viable yeast cells.

The alcohol percent produced was found to be 6.7% (v/v); almost the same produced at the end of the control lager fermentations (6.39%). Such an effect shows that even after 212h of fermentation, the same effect was induced in terms of ethanol production.

The total dry biomass was found to be similar to the resultant dry yeast crop collected at the end of the control fermentations. It appeared (as would be expected) that ammonia did not to have a stimulatory effect on yeast cell division and additionally such an extended sugar attenuation period (212h) had a negative effect on the viability of yeast cells compared to the control fermentations, since it decreased to 90%.

3.3.6 Vicinal diketones and their precursors during the supplemented fermentations

Figure 70 presents the VDKs and their precursor concentrations produced during all wort supplemented fermentations. The final VDK levels formed during the control fermentations are also shown in **Figure 70** for comparison.

The levels of diacetyl, 2,3-pentanedione and their precursors formed during the fermentations with added lysine were approximately double those produced during the control fermentations. On the other hand, during methionine-supplemented fermentations, the concentrations of these flavour compounds and their precursors were lower than the control. By examining the fermentations in which arginine was added as a nitrogen wort supplement, it was found that the final VDK levels produced in beer were elevated compared to that measured during the unsupplemented fermentations. The same effect was also observed when the final concentration of the precursors of the vicinal diketones were measured. Finally, in the fermentations which were supplemented with additional ammonium ions, it was found that the final VDKs and their precursor content was less than that produced during the control fermentations.

As discussed above, lower diacetyl and 2,3-pentanedione levels were present in the fermented worts supplemented with methionine and ammonia than in the control fermentations. These lower levels could be due to prolonged fermentation times in the presence of excess concentrations of methionine and ammonium ions. Decreased yeast growth could also provide an explanation for the relatively low levels of VDKs encountered at the end of these fermentations. On the other hand, the excessive biomass production is likely responsible for the elevated VDK levels of the supplemented fermentations with lysine and arginine.

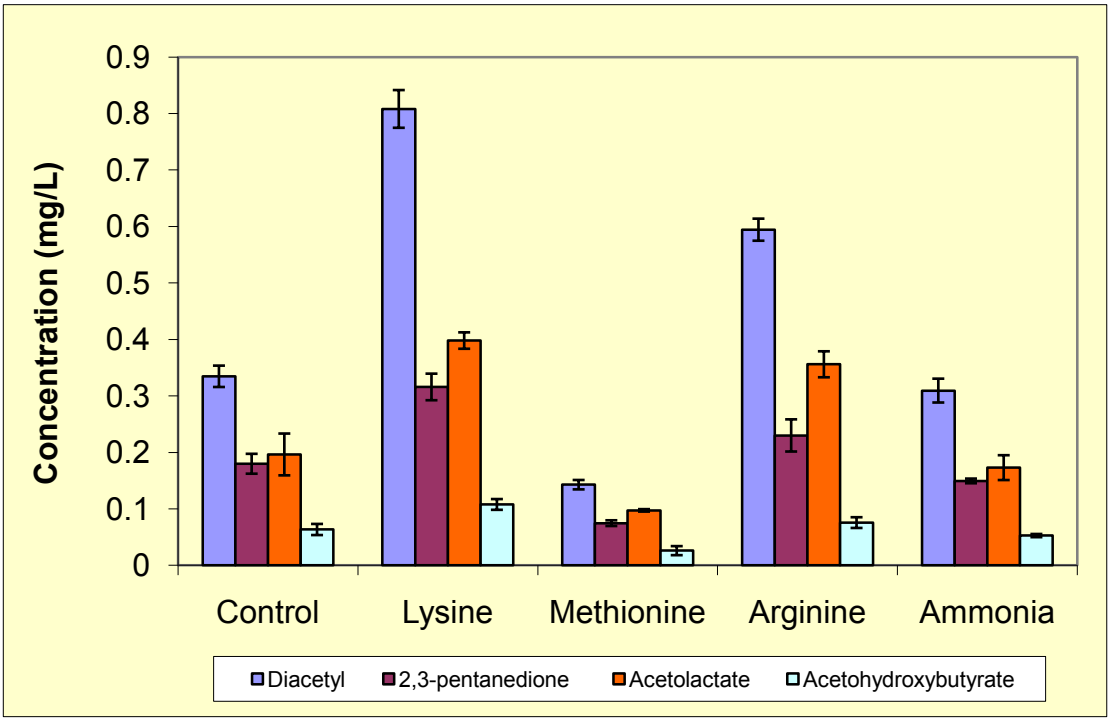


Figure 70: Final vicinal diketones levels and their precursor concentrations for the SC3 lager supplemented fermentations conducted with five times more lysine, methionine, arginine and two times more ammonia. The final VDK levels and their precursor concentrations are also plotted for the unsupplemented fermentations (control) for comparison purposes. The data presented are the mean values of three fermentations ± S.D.

3.4 Amino acid supplementations using statistical experimental design

The amino acid and ammonia spectrum concentrations, in the high gravity adjunct wort (15^{o}Plato, 70% malt and 30% VHM syrup) used for all the static fermentation experiments were analysed using the statistical program Minitab (Minitab 14 statistical software, Minitab Ltd, Coventry, UK). The statistical software analysed the provided data and it prepared the experimental design and the number of all possible amino acid and ammonia wort supplementation combinations, with concentrations twice the initial concentration of the natural nitrogenous wort constituents that can be met. Minitab was used to design unified supplementation experiments because single amino acid and ammonium ion supplementations did not provide any valuable information concerning synergistic effects or interactions of the yeast's available nitrogen sources or information about the ideal wort nitrogen compound composition for producing the same quality and stability beer in less fermentation time.

During the fermentations, wort sampling was carried out, at set time intervals for measuring the specific gravity of the fermenting wort. When the target gravity for each individual fermentation cylinder (3^{o}Plato) was reached, the experiment was terminated. Additionally, final fermented wort samples from each experiment were analysed for the spectrum of beer's volatile compounds in order to study how the enrichment of the fermentation medium with extra assimilable nitrogen affected the flavour and stability of the final product.

The data from the fermented wort and beer flavour compound concentrations collected from the 25 experiments was used and by conducting a new statistical analysis, a plethora of interesting results was developed, but only a selective sample number of them is shown here.

3.4.1 Time to target PG (3°Plato)

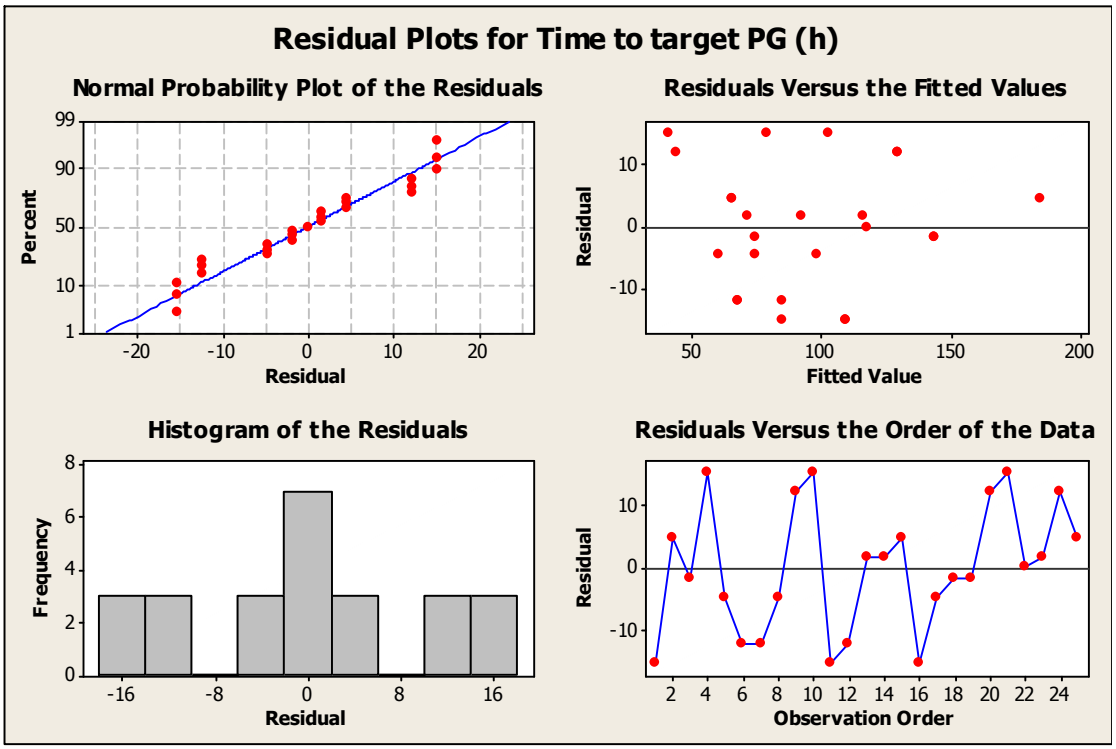


Figure 71: Residual plots for the time taken for each fermentation to reach the target present gravity (3°Plato).

The collected time data taken for each supplemented fermentation to reach the desired present gravity was statistically analysed in order to study the difference in yeast metabolic behaviour for the lager strain SC3, with the 25 different nitrogenous material combinations. The first set of residual plots (**Figure 71**) is the complex analysis test that reveals statistical assumptions. To be more specific, the Normal Probability plot detects non normality. An approximately straight line indicates that the residuals are normally distributed. The Histogram of the Residuals detects multiple peaks, outliers, and non normality. The histogram should be approximately symmetric and bell-shaped. The Residuals versus the Fitted Values plot detects non constant variance, missing higher order terms, and outliers. The residuals should be scattered randomly around zero. Finally, the last plot of the complex, the Residuals versus Order detects time dependence of the residuals. The residuals should exhibit no clear pattern. Hence, having all the above mentioned information in mind, **Figure 71** reveals that the residuals are normally and randomly distributed. Also the observation order had no influence on the results.

The next figure (**Figure 72**), which is called a "Pareto Chart of the Effects", determines the magnitude and importance of an effect (Minitab 14 statistical software). The chart displays the absolute value of the effects and draws a reference line on the chart. Any effect that extends past this reference line is potentially important. The program displays the absolute value of the effects on the Pareto chart. Any effects that extend beyond the reference line are significant at the default level of 0.05. Hence, **Figure 72** shows the relative significance of each amino acid and ammonia on the completion fermentation time, with lysine having the greatest influence over the time taken to reach target PG and serine having the least influence. Moreover, the increase in the lysine wort concentration was found to have induced the only significant effect on the time needed for the yeast to metabolise the fermentable sugars and reach the desired final gravity.

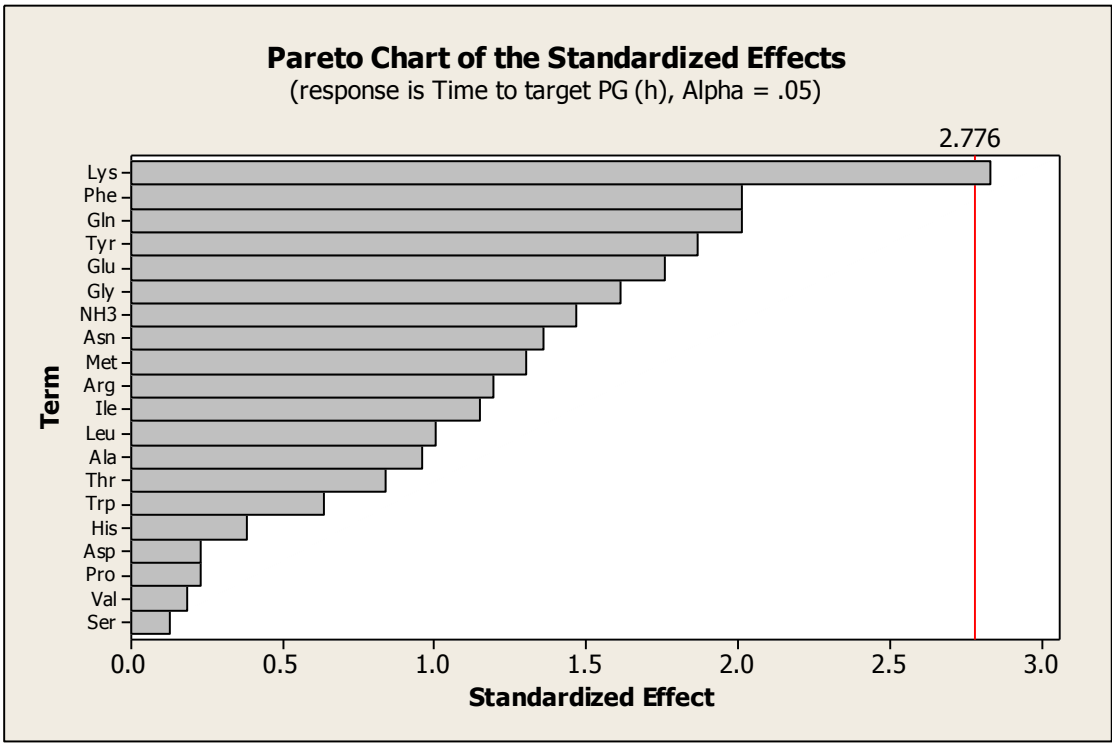


Figure 72: The importance of nitrogenous materials on the time to target PG

Figure 73 depicts the Normal Probability plot of the effects, the points that do not fall near the line determine important effects. Important effects are larger and further from the fitted line than unimportant effects. Unimportant effects tend to be smaller and centered around zero. The normal probability plot uses $\alpha = 0.05$, by default. Based on this explanation, **Figure 73** confirms the results shown on the Pareto chart that lysine was

the only amino acid, which had a significant effect on yeast fermentative efficacy. In other words, lysine was found to be the key amino acid that triggers a faster fermentation compared to the control experiments conducted with the same wort type and yeast strain. Therefore, Minitab provides these two graphs the Normal probability and the Pareto chart in order to compare and identify the relative magnitude of the influential factors and evaluate their statistical significance.

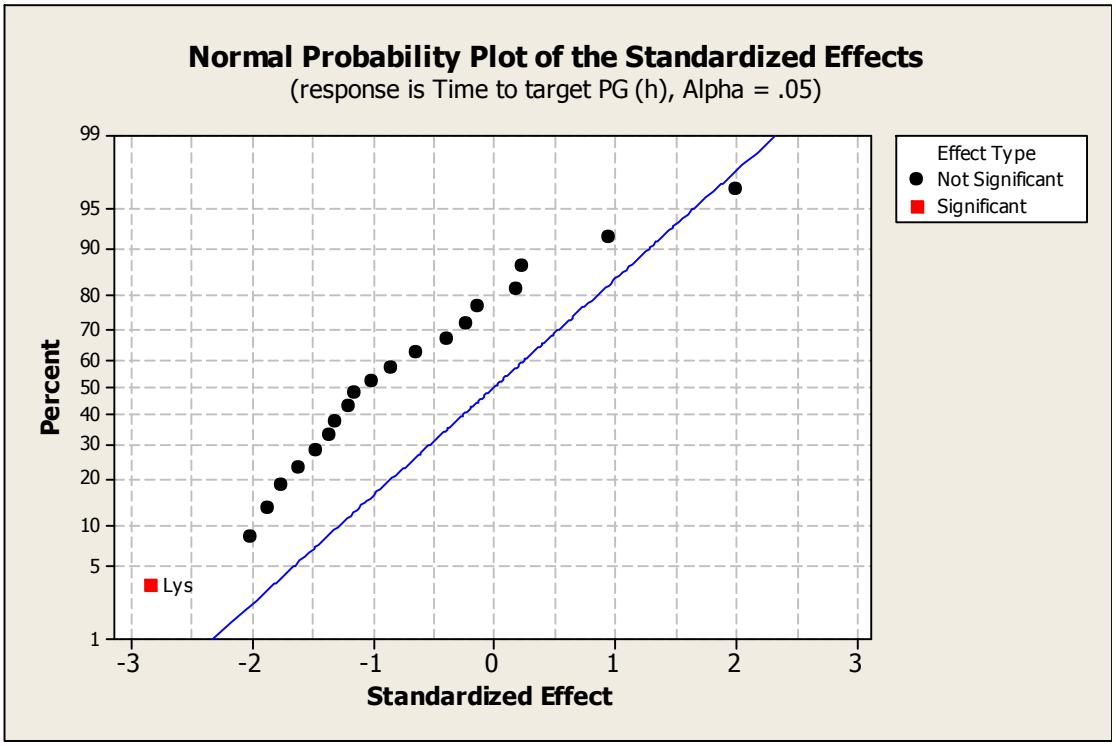


Figure 73: Lysine increase in wort had the only significant effect on the fermentation rate

Figure 74 is known as the Main Effects Plot and identifies the relative effect that each individual nitrogenous wort material has on the rate of fermentation. In more detail, the nature of the actual effect of each amino acid and ammonium ions is illustrated in **Figure 74**. The steeper the line, the greater the influence of the effect that the amino acid has on yeast metabolic activity. The steepest line in the Effects Plot was lysine indicating, once again, that the increase in wort lysine concentration had the most important and influence on the rate of the fermentation (by 95%). The centre points do not fall between the corner points indicating non-linearity of data.

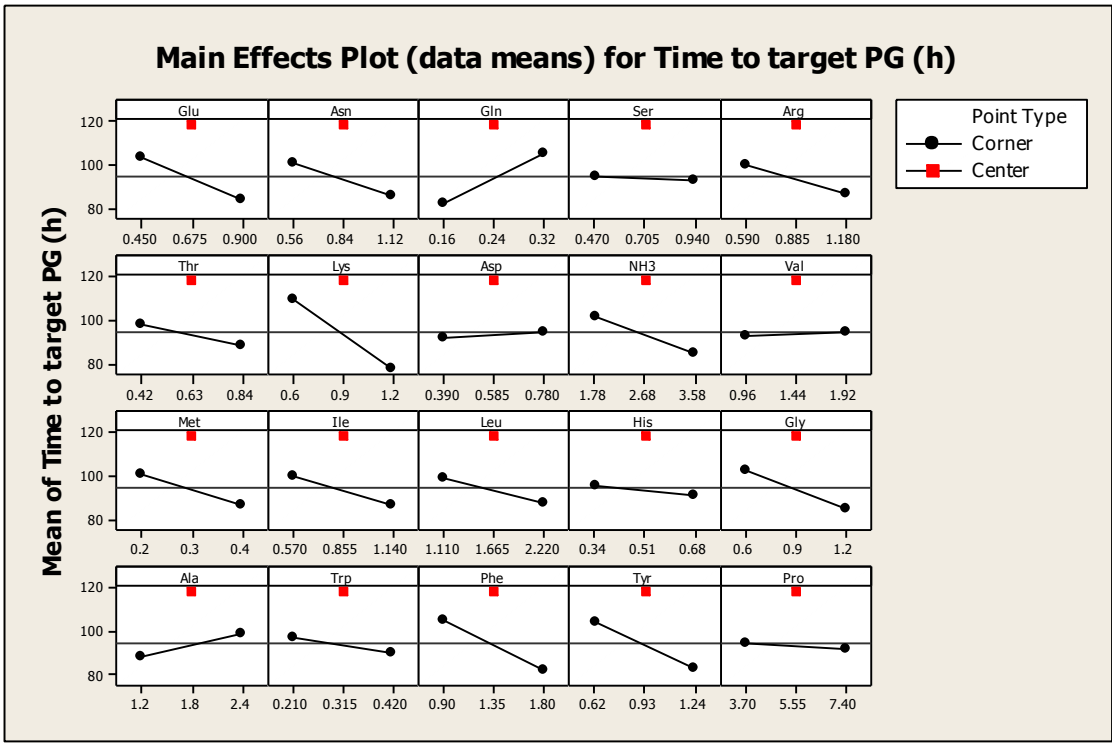


Figure 74: The effect that the increase of each individual wort nitrogen constituent had on the time to target gravity

Table 21: The effect of the increased amino acid concentration on the wort completion time

Faster fermentation	No change	Slower fermentation
Glu	Ser	Gln
Asn	Asp	Ala
Arg	Val	
Thr	His	
Lys	Pro	
NH_3		
Met		
Ileu		
Leu		
Gly		
Phe		
Tyr		

As it can be seen from **Table 21**, the increase in the initial wort concentration of the amino acids serine, aspartic acid, valine, histidine and proline did not induce any effect on

the rate of fermentation. Glutamine and alanine seem to be the two amino acids whose increased concentration inhibits the yeast cells fermentative performance and hence the fermentation becomes sluggish and slower than the control. Finally, all the other amino acid and ammonia wort enrichments seem to have enhanced yeast metabolic activity and simultaneously reduced the time needed to reach the target sugar attenuation level. Minitab provides an option to identify the combination and interaction of input variable settings that jointly optimize a set of responses. These commands can be used after you have created and analyzed factorial designs. Thus, by creating an optimisation plot, the optimal amino acid concentrations can be calculated in order for the fermentation to be completed within the fastest possible time. The optimization plot is interactive; meaning that the input variable settings on the plot can be adjusted in order to search for more desirable solutions.

Figure 75 illustrates an optimisation plot in which the individual desirability for minimum time taken to reach target gravity is 1, which is the optimal desirability value. The sum of the effects that each individual amino acid and ammonia supplementation had on the fermentation completion time was statistically analysed and the preferably nitrogenous material wort content for achieving the best yeast fermentative performance calculated.

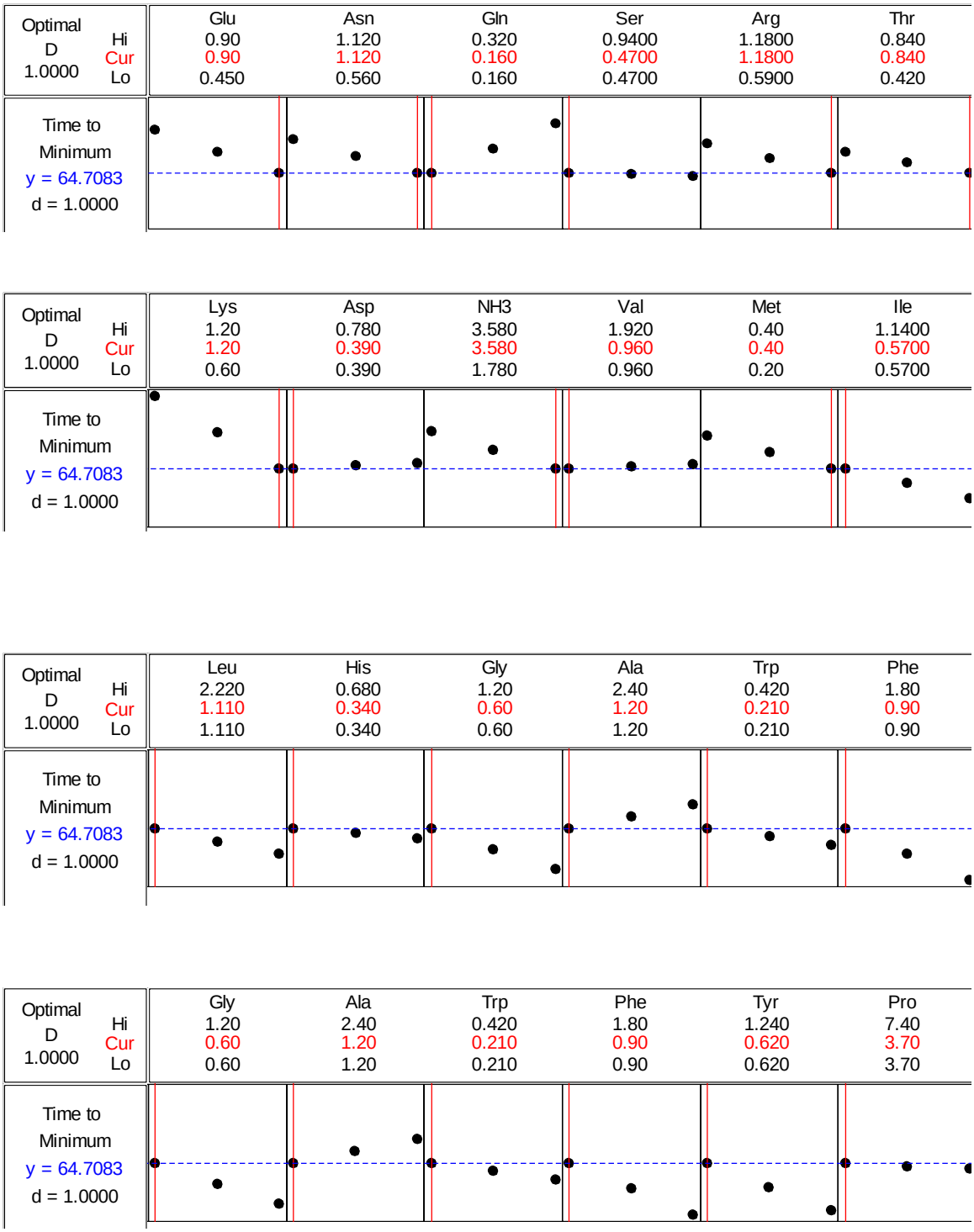


Figure 75: The optimal synergistic nitrogen wort content combination for fermentation completion

Table 22: The optimal wort nitrogen consistency for the fastest possible fermentation

Nitrogen compound	Concentration (mmoles/L)	Nitrogen compound	Concentration (mmoles/L)
Glu	0.90	Asn	1.12
Gln	0.16	Ser	0.47
Arg	1.18	Thr	0.42
Lys	1.20	Asp	0.39
NH_3	1.78	Val	1.92
Met	0.40	Ile	0.57
Leu	2.22	His	0.68
Gly	0.60	Ala	1.20
Trp	0.21	Phe	0.90
Tyr	1.24	Pro	3.70

This wort nitrogenous composition (**Figure 75 & Table 22**) calculated by the appropriate software was found to be the optimal amino acid and ammonia synergistic wort combination in order for the fermentation to be completed in the minimum possible time, which was found to be 65h. Hence, if the yeast strain SC3 is pitched in an adjunct wort with original gravity 15^{o}Plato and with the nitrogenous material constituency listed in **Table 22**, it is believed that the fermentation will be terminated after 65h of yeast incubation. Thus, the total operation time for an industrial scale beer production using this enriched nitrogen wort type, will decrease by approximately 30h since the fermentation time needed for a control lager SC3 fermentation was 96h.

3.4.2 Total butanedione production

Figure 76 shows the statistical analysis conducted for the total diacetyl composition produced at the end of the supplemented fermentations. The plotted data appears to be acceptable for analysis even though the Histogram of Residuals is not perfectly shaped as it is not in the shape of a normal distribution (bell-shaped).

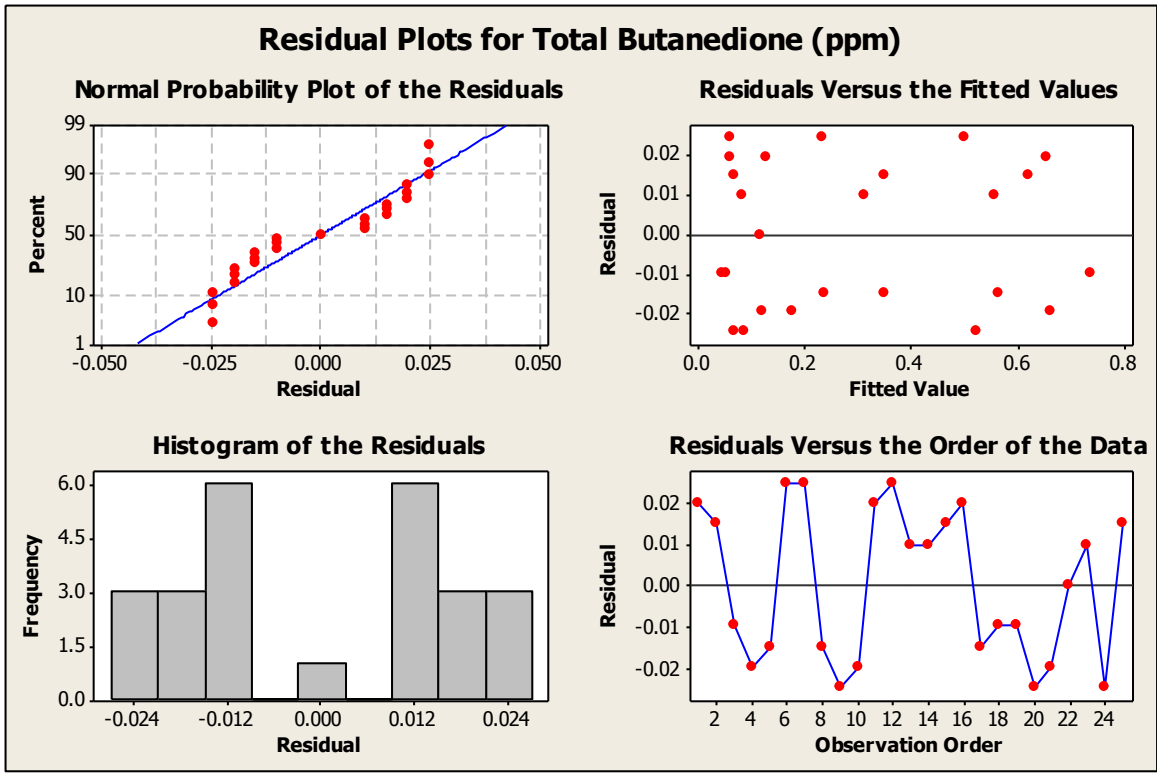


Figure 76: Residual plots for the production of total diacetyl at the end of fermentations

Both the Pareto Chart and the Normal Probability Plot (**Figures 77 & 78**) indicate that the increase in valine concentration had the most important effect on the final diacetyl production and that arginine increase did not influence the final levels of diacetyl found in beer.

Additionally, from the spectrum of all nitrogenous wort compounds examined, valine, lysine, leucine and histidine had a significant influence on the production of butanedione, meaning that the increase of these particular wort nitrogen constituents had affected the diacetyl production by 95% probability.

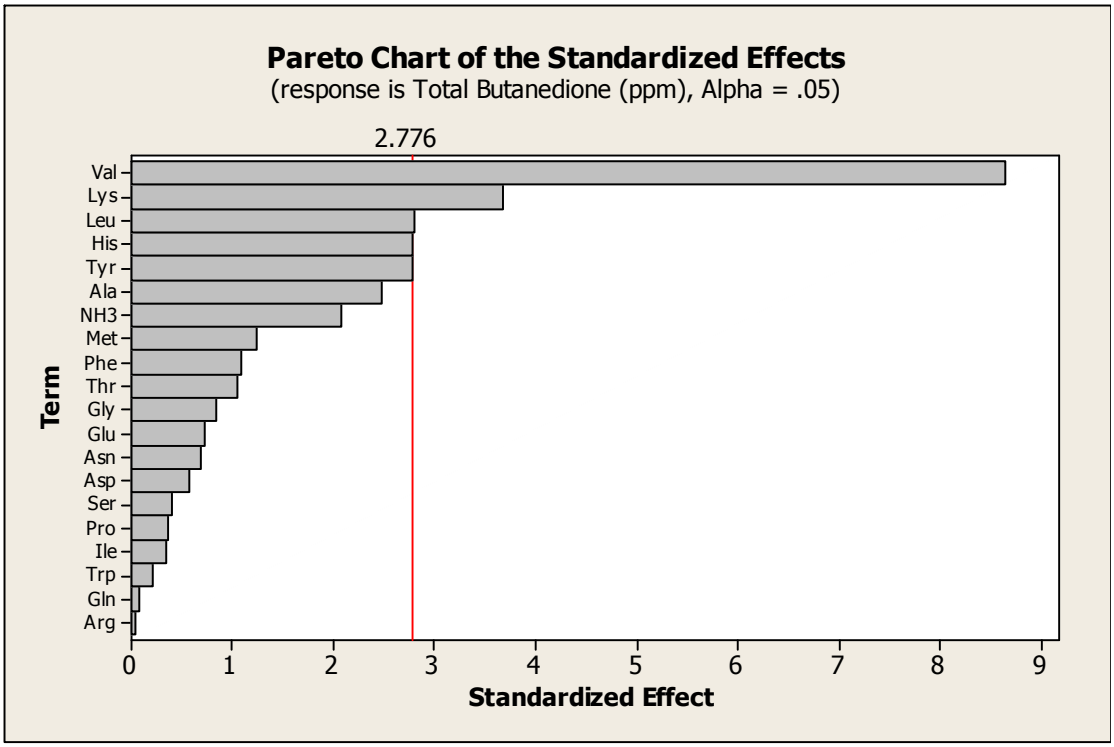


Figure 77: The degree of the effect that each individual nitrogen compound had on the production of diacetyl

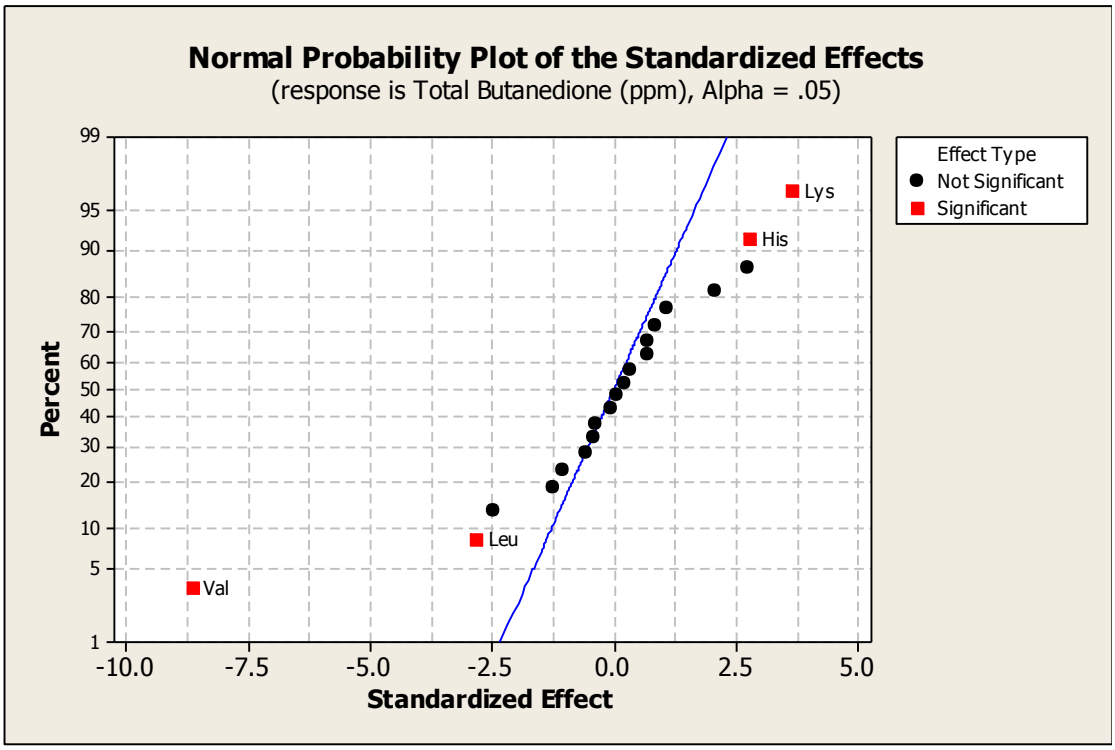


Figure 78: Lysine, histidine, leucine and valine were the only amino acids that had a significant effect on the diacetyl production

The above experimental statistical model can be simplified by removing all the non-significant terms in order to have a more understandable idea about the importance of these four different sources of yeast's assimilable nitrogen. Thus a new version of Residual Plots (**Figure 79**) was prepared without taking account of the non-significant factors. Such an action assisted the improvement of the appearance of the Histogram of the Residuals indicating that a better model had been obtained since it converted it into a bell shaped histogram.

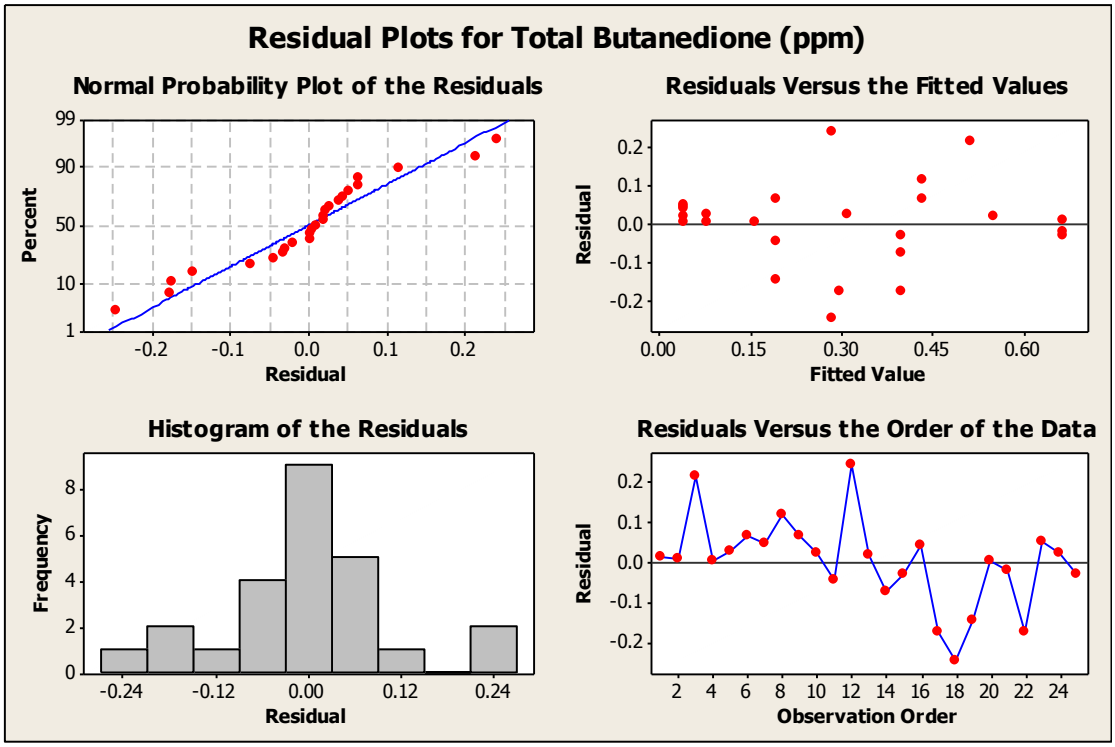


Figure 79: The Residual Plots for the total diacetyl production after the exclusion of the non significant factors

A better version of a Pareto Chart of the significant amino acids for the production of diacetyl is illustrated in **Figure 80**. The link between butanedione formation and the concentrations of valine and leucine are well known by many various publications (e.g. Holmberg and Petersen, 1988; Petersen *et al*. 2004; Xiao and Rank, 1990). Lysine and histidine were not found to have been reported as significant factors on the formation of diacetyl in the final beer.

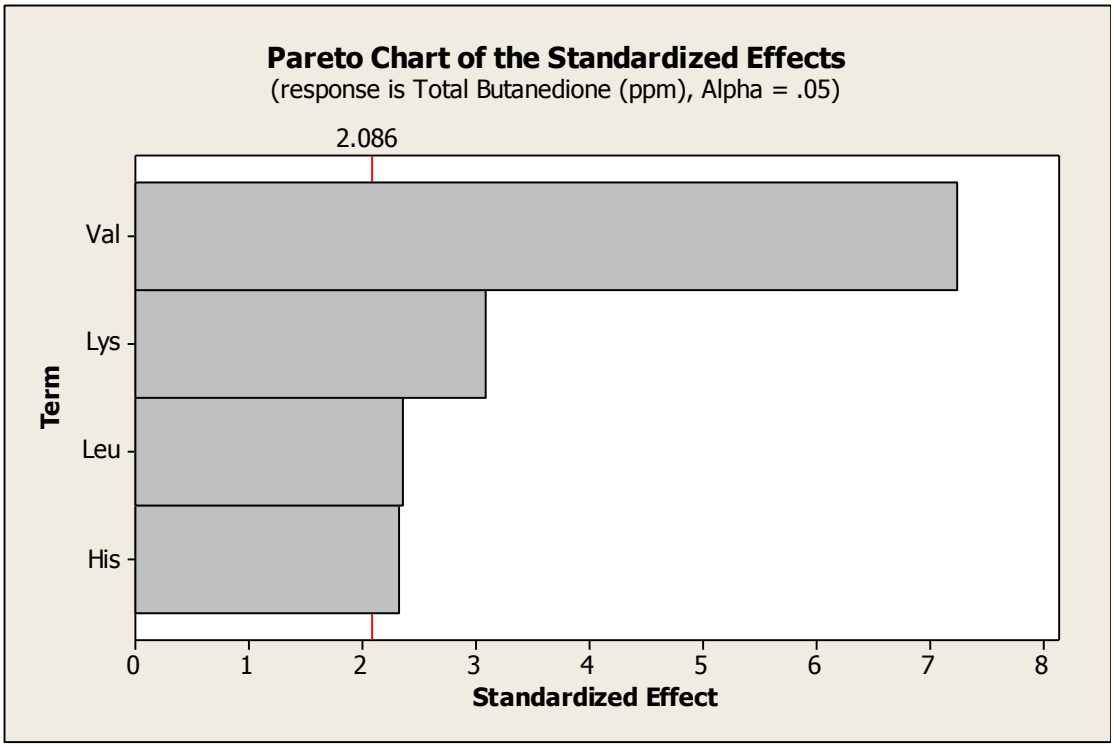


Figure 80: The main amino acids that have a significant effect on the total diacetyl production

With this new reduced model, the main effects plot (**Figure 81**) was constructed. The increase in the lysine and histidine concentrations elevated the final diacetyl concentration, exactly as occurred at the end of the X5 lysine supplemented fermentations, where elevated levels of diacetyl were detected. On the other hand, with the increase in the valine and leucine concentrations in the fermentation medium, the final diacetyl levels decreased. Valine according to the angle of its plot line seems to be the most important amino acid that affects the total concentration of butanedione at the end of fermentations. The diacetyl and pentanedione precursor metabolites, α-acetolactate and α-acetohydroxybutyrate respectively, are produced during fermentation by yeast as intermediates during biosynthesis of the branched amino acids isoleucine, leucine, valine via the regulated ILV pathway (Holmberg and Petersen, 1988). The overall pathway activity, and hence precursor and VDK formation, is partly affected by the concentration of isoleucine, leucine and valine present in the wort (Holmberg and Petersen, 1988; Petersen *et al*. 2004; Xiao and Rank, 1990). The presence of high levels of valine in the wort have been noted to be a factor linked to reduced diacetyl evolution during fermentation (Petersen *et al*. 2004). In addition, Owades *et al.* (1959) reported that valine

suppresses the formation of diacetyl. Low valine concentration in unfermented wort results in valine rapid depletion during fermentation and high concentrations of diacetyl in the final beer (Owades *et al.* 1959). The formation of α-acetolactate (diacetyl's precursor) is regulated by both the valine and the total wort FAN concentration (Owades *et al.* 1959).

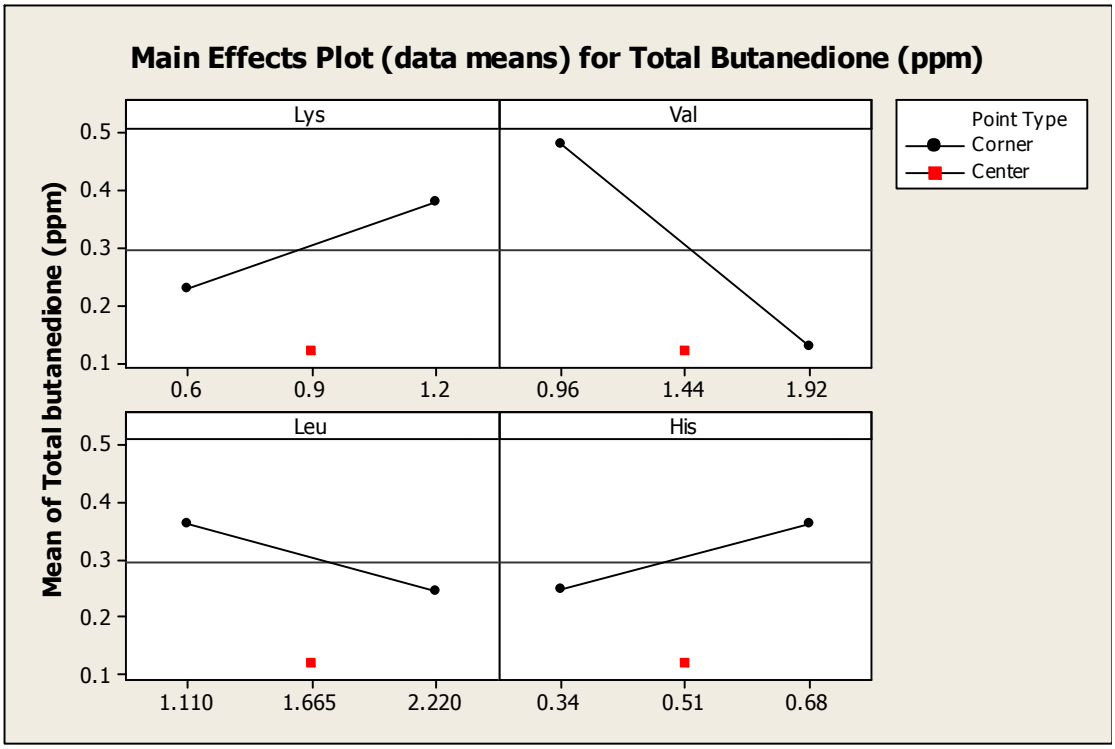


Figure 81: The effect of the increase of lysine, valine, leucine and histidine on the production of total diacetyl

3.4.3 Total pentanedione production

A similar analysis for pentanedione can be made in the same way that diacetyl was statistically analysed by Minitab. **Figure 82** illustrates the Residual plots for total pentanedione and as it can be seen, the residuals are normally and randomly distributed. Also the observation order had no influence on the results.

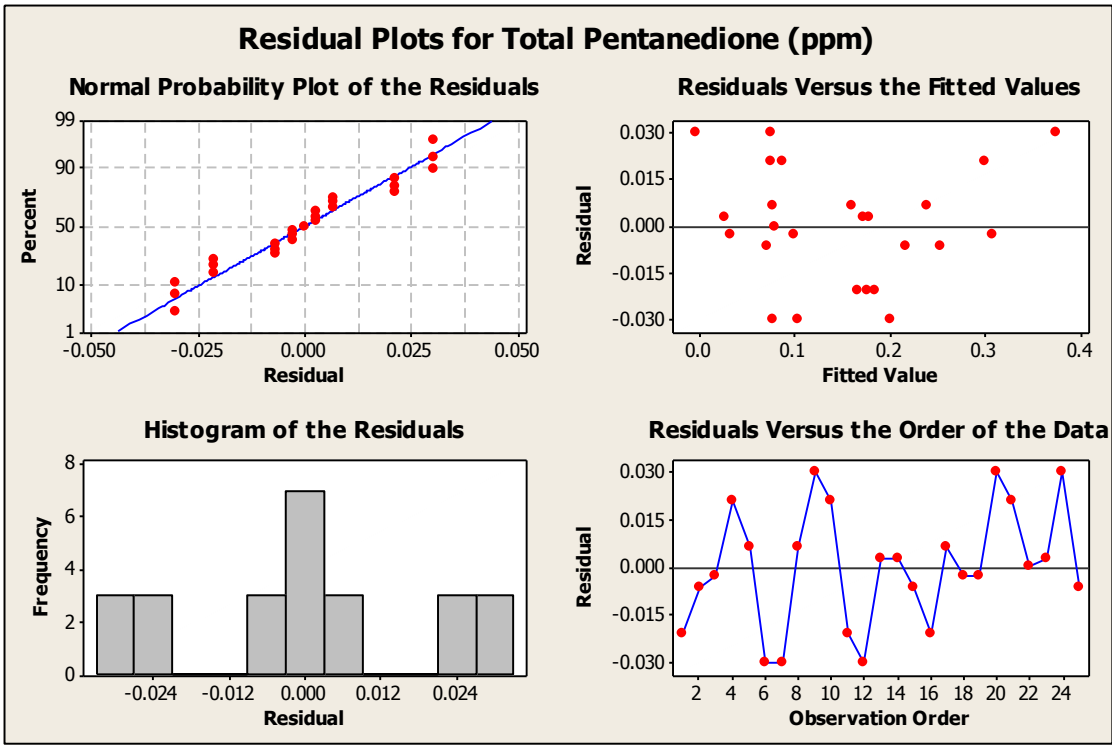


Figure 82: Residual plots for the production of total pentanedione at the end of fermentations

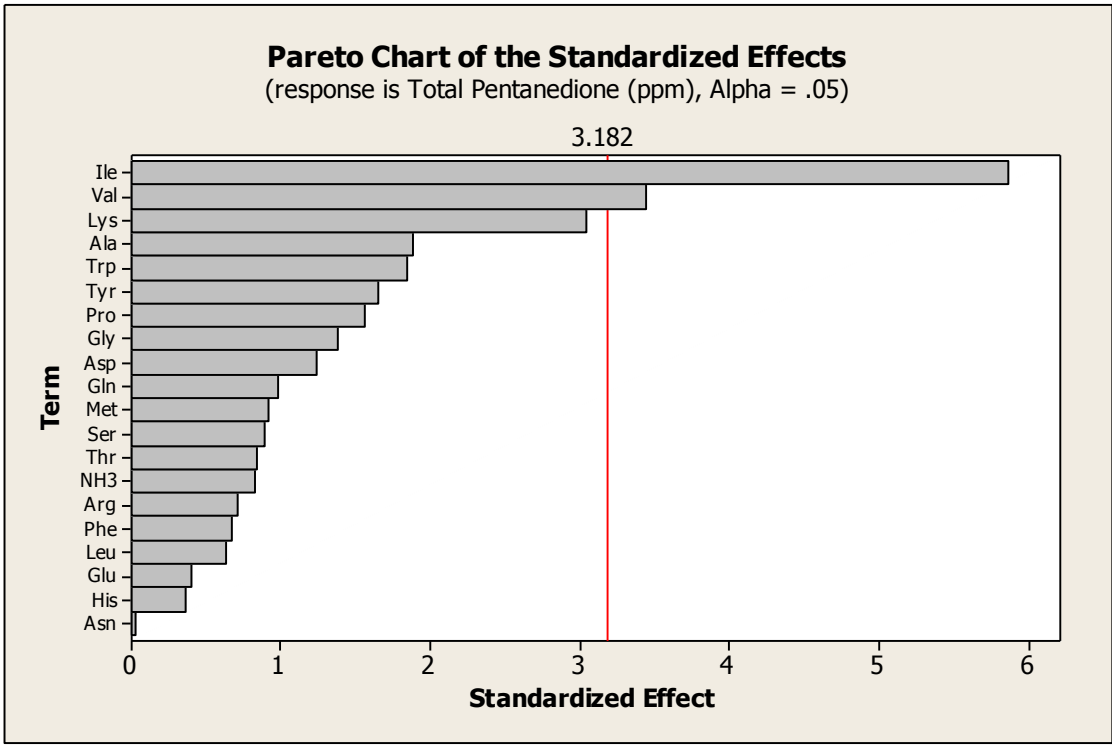


Figure 83: Amino acids that have a significant effect on the total pentanedione production

Pareto Chart (**Figure 83**), an isoleucine increase in the wort had the largest effect on the pentanedione formation and asparagine was found to be the amino acid whose increase in concentration did not play any role at all in the formation of total pentanedione.

All the non significant factors for affecting the total pentanedione formation were excluded and a new more simplified version of Pareto chart was developed (**Figure 84**). The significant factors, which are isoleucine and valine shown in the Pareto Chart, are the two amino acids that have to be analysed as how they influence the total pentanedione concentration.

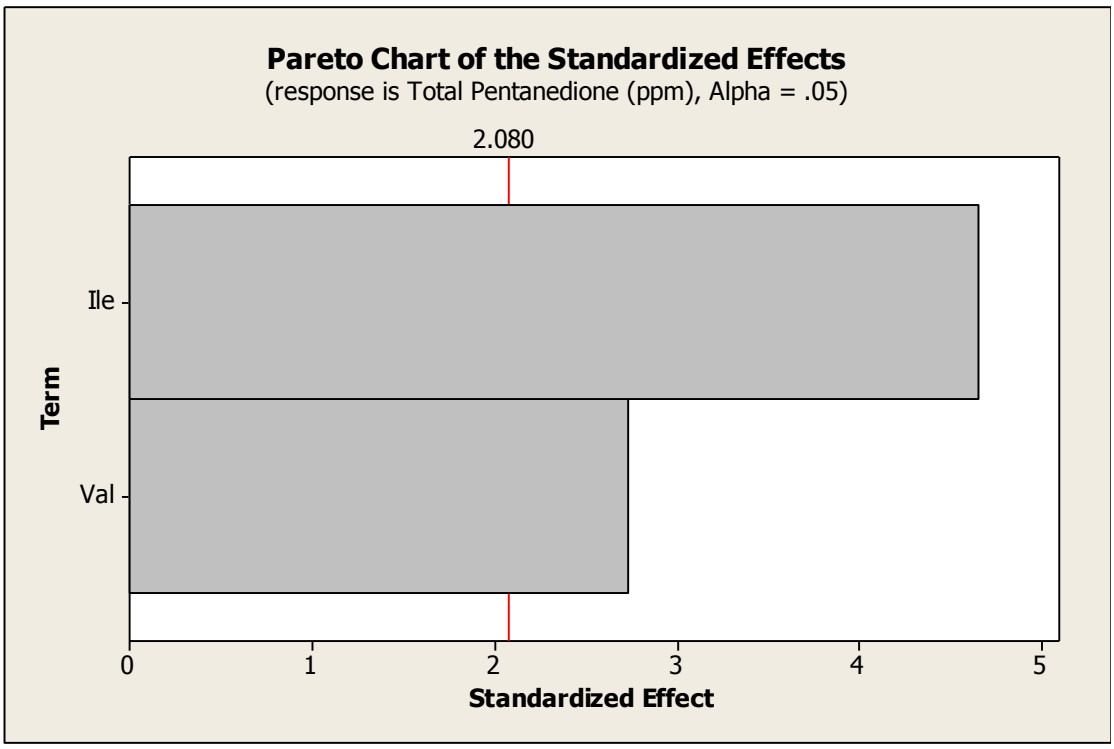


Figure 84: Simplified Pareto Chart after excluding the non significant factors affecting the total pentanedione production

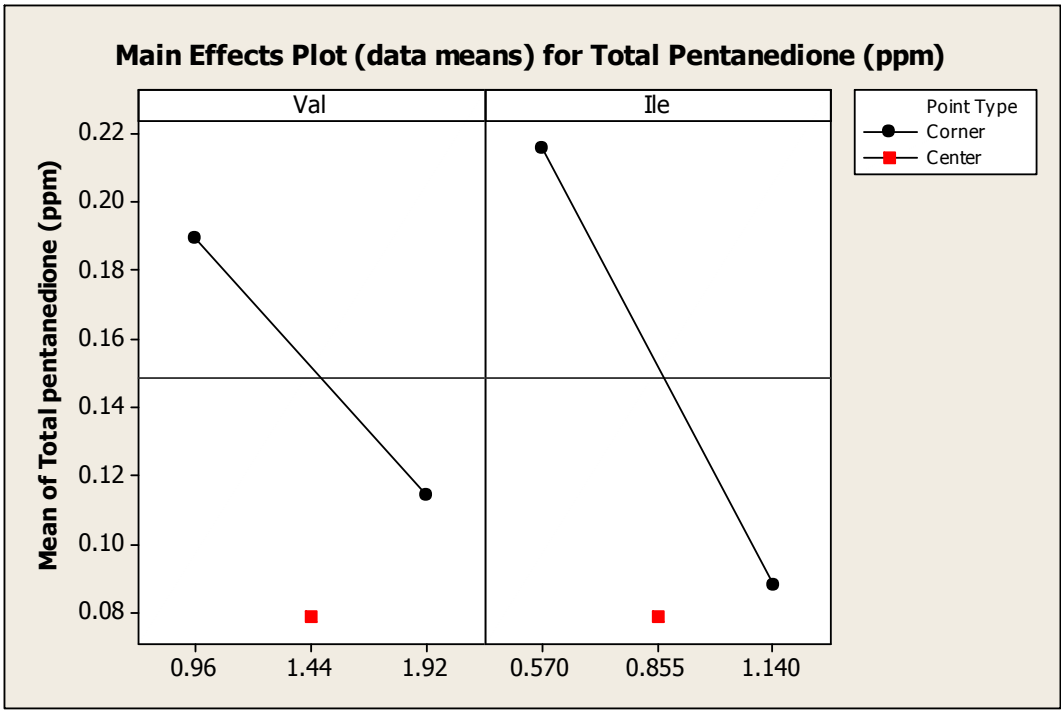


Figure 85: The effect that both valine and isoleucine have on the total pentanedione formation

As it can be understood from the Main Effects Plot for total pentanedione (**Figure 85**), the increase of valine and isoleucine concentration from their original concentration found in the fermentation medium into double the amount of this concentration causes a decrease in the concentration of pentanedione, as already reported in the literature (Holmberg and Petersen, 1988; Petersen *et al*. 2004; Xiao and Rank, 1990).

3.4.4 Total acetylaldehyde production

A similar statistical analysis was chosen to be conducted for acetylaldehyde since it is a very important beer flavour compound affecting the quality of the final product. The formation of total acetylaldehyde over its threshold value (10ppm) in beer, gives rise to off flavours of freshly cut grass and green apples (Bamforth, 2000).

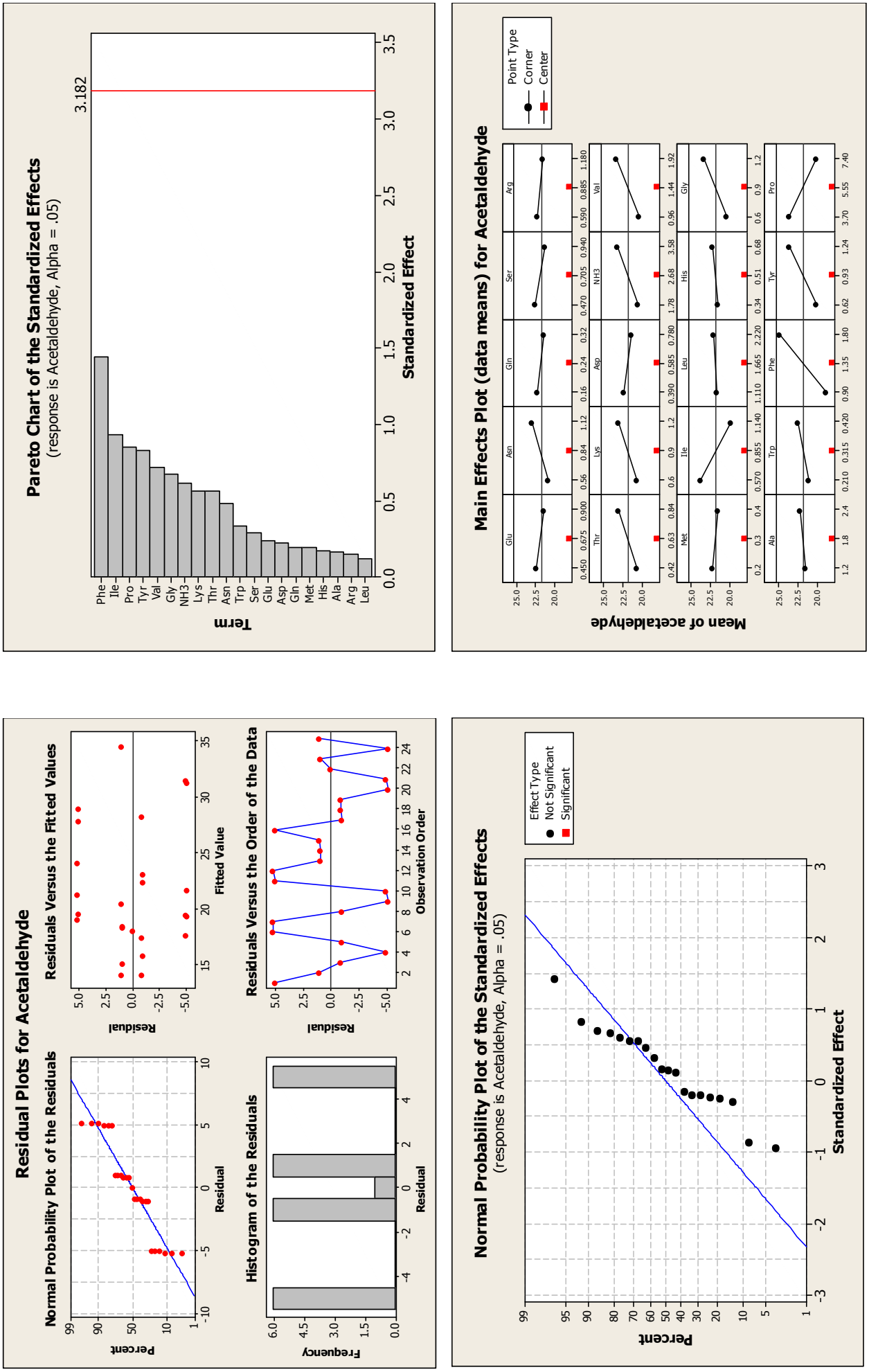


Figure 86: Overall statistical analysis for the total acetylaldehyde formation

Figure 86 illustrates the statistical analysis of total acetylaldehyde formation, the Pareto Chart shows the relative significance of each amino acid, with phenylalanine having the greatest influence on the formation of total acetylaldehyde and leucine having the least influence. There is, however, a problem with this data. None of the data is considered to be significant at the 95% confidence level and this observation is also confirmed by examining the Normal Probability plot. Hence, none of the increase of nitrogen wort constituents had any significant effect on the production of acetylaldehyde.

Table 23: The effect of the increased amino acid concentration on the formation of total acetylaldehyde in beer

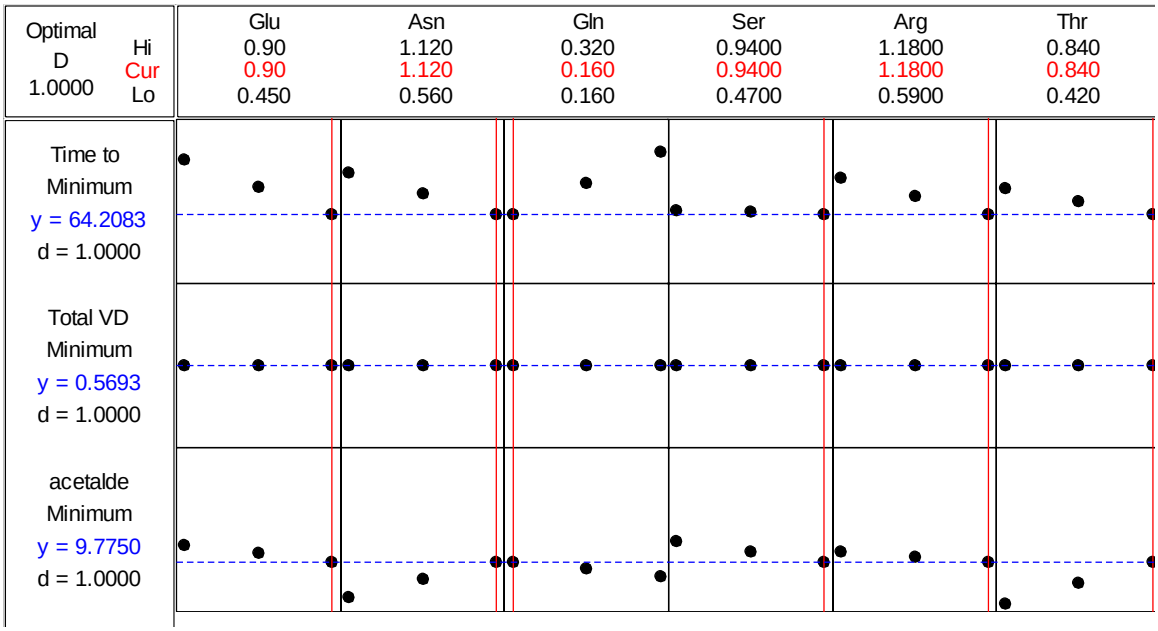
Increase on acetyladehyde formation	**No change on acetyladehyde formation**	**Decrease on acetyladehyde formation**
Asn	Gln	Glu
Thr	His	Pro
Lys	Meth	Ser
NH_3	Arg	Asp
Val	Leu	Ile
Gly	Ala	
Phe		
Tyr		
Trp		

Since each statistically examined factor (attenuation rate, total VDK's and total acetylaldehyde) is important in determining the quality of the final product, we need to take under consideration and these three individual factors simultaneously.

By running a combined Optimisation test for these three different factors (**Figures 87a & 87b**) it was found that the composite desirability for all these variables is 1, which is the optimal desirability value. Thus, in order to obtain this combined three variables optimal desirability, the following amino acid values (**Table 23**) should be contained in the initial wort medium prior to yeast pitching. If the following nitrogen wort composition is available for the yeast cells, then the time needed to reach the desired target gravity fermentation will be 64h, thirty two hours faster than the control fermentation. Moreover, as it can be seen in both **Figures 87a & 87b** of the Optimisation Test plots, the minimum value for total VDKs will be 0.57ppm, if the statistically calculated amino acid and ammonia composition is contained in the unfermented wort. This total VDK value is less

than the VDK taste threshold value in final beer, which is 1.05ppm (0.15ppm for diacetyl and 0.9ppm for 2,3 pentanedione). VDK levels over this value give raise into the undesirable effects of butterscotch flavour. The same observation is also valid for total acetylaldehyde, whose estimated produced value will be 9.7ppm, less than its taste threshold value (10ppm) with the statistically analysed wort nitrogen sequence. Acetylaldehyde levels over its threshold value in final beer produce off flavours of fresh cut green apples or cut grass.

Finally, the statistical analysis of these three important beer quality and stability factors can be considered as a very useful tool for the brewer since by analysing the amino acid and ammonia spectrum of the malt variety under standard mashing and fermentation conditions (yeast strain, temperature, fermenter geometry, wort gravity, adjunct levels etc.) prior to fermentation, they would be able to have a very accurate prediction for the duration of the fermentation and the quality of the produced beer. By knowing the nitrogen composition of the malt, brewers can choose the most compatible with the yeast strain in order to establish the best case scenario in terms of fermentation time and beer quality and stability.



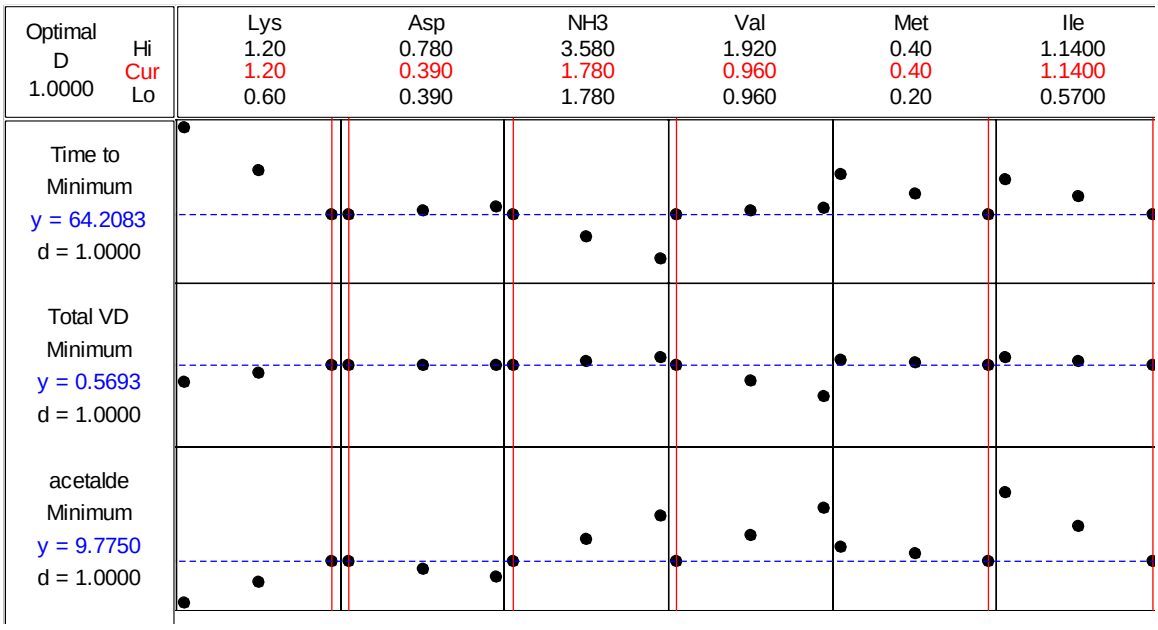
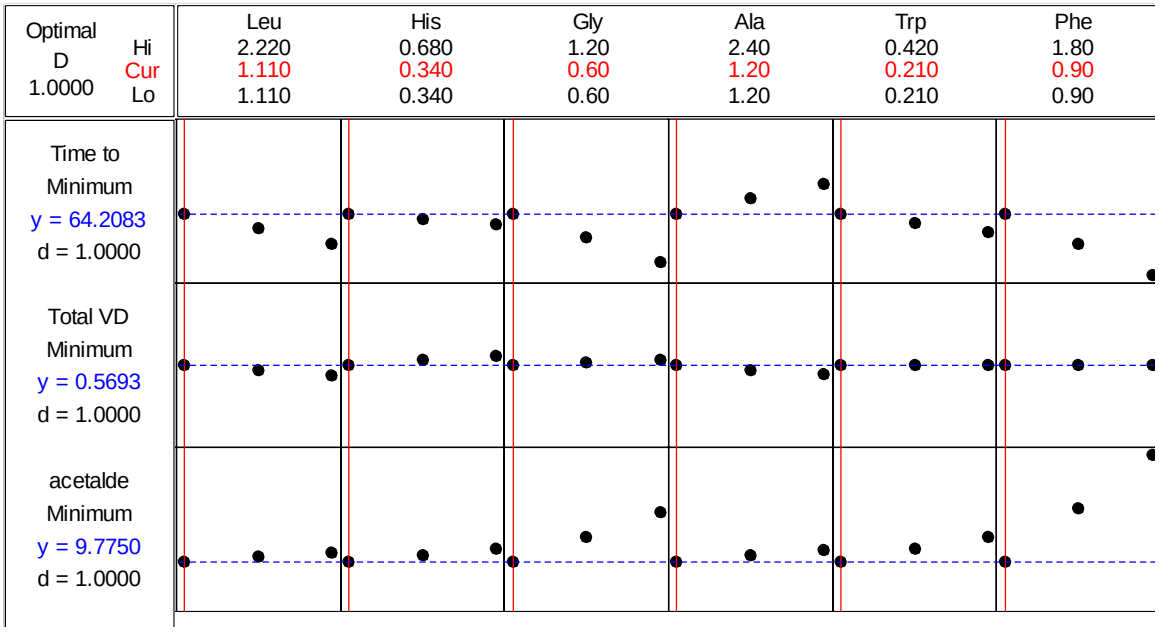


Figure 87a: The optimal wort nitrogen composition for achieving the fastest fermentation rate and the minimum concentrations for total VDK's and acetylaldehyde formation



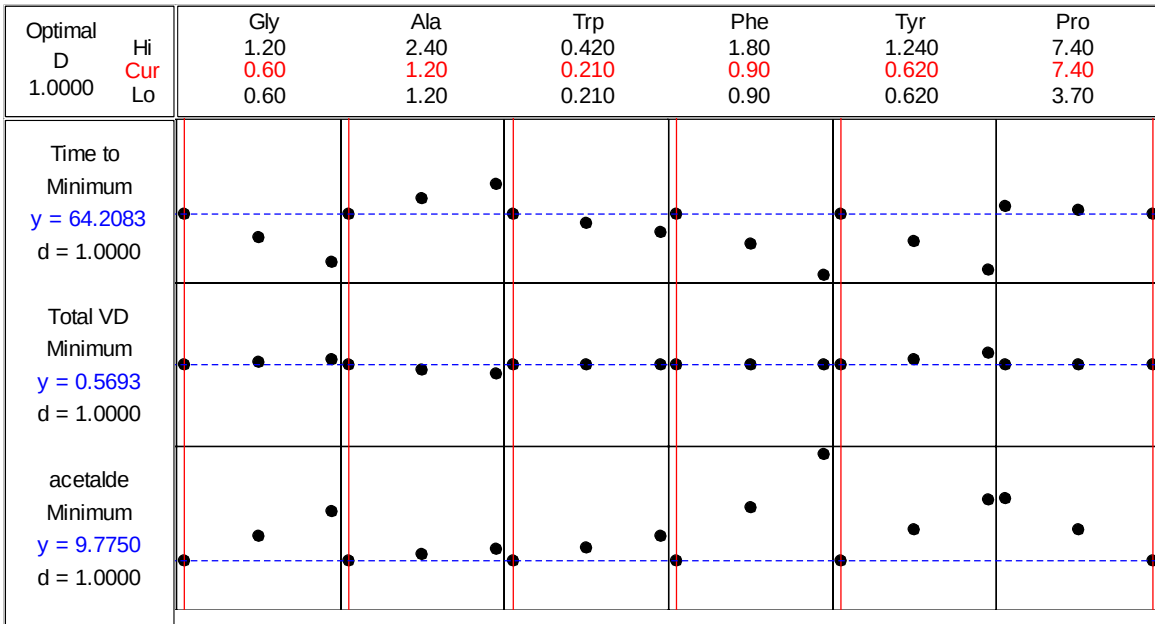


Figure 87b: The optimal wort nitrogen composition for achieving the fastest fermentation rate and the minimum concentrations for total VDK's and acetylaldehyde formation

Table 24: The optimal wort nitrogen consistency for the fastest possible fermentation providing minimum levels of VDK's and acetyladehyde

Nitrogen compound	Concentration (mmoles/L)	Nitrogen compound	Concentration (mmoles/L)
Glu	0.90	Asn	1.12
Gln	0.16	Ser	0.94
Arg	1.18	Thr	0.84
Lys	1.20	Asp	0.39
NH_3	1.78	Val	0.96
Met	0.40	Ile	1.14
Leu	1.11	His	0.34
Gly	0.60	Ala	1.20
Trp	0.21	Phe	0.90
Tyr	0.62	Pro	7.40

3.5 Oligopeptide determination

The novel method that was developed in order to detect, isolate and measure small peptides with molecular weight less than 500 Daltons, was described in detail in the Materials and Methods Section (***2.3.9***). Due to the fact that this technique had not be evaluated in terms of its validity and reproducibility by other researchers, only three fermentations were chosen to be examined three times each for confirming how valid were the results. The fermentations that were selected to be studied for oligopeptide nitrogen metabolic behaviour were a normal gravity (12^{o}Plato) ale (No 70) shake flask fermentation and an ale (SC8) and a lager (SC3) yeast static fermentations. Therefore, these three different fermentation experimental conditions were repeated in order to analyze the spectrum of wort small peptides. This novel assay was not tested using known concentrations of one or more oligopeptides.

Figures 88, **89** and **90** illustrate the overall ale shake flask and the static lager and ale yeast fermentation nitrogen metabolic profile, respectively. The sum of the spectrum of all wort amino acids is also shown in these figures, together with ammonia and proline. Such a plot orientation obliges a comparison of the metabolic rates of all the available wort nitrogenous yeast sources during the fermentation progress. During the ale shake flask experiments, the uptake of wort oligopeptides commenced rapidly after the first day of fermentation and it lasted for 24h. A significant increase in the levels of small peptides was detected in the fermenting wort, between 48 and 72h of yeast incubation. Thereafter, the oligopeptide wort content was utilized once more, while during the last 24h of fermentation its concentration started to increase in the fermentation broth.

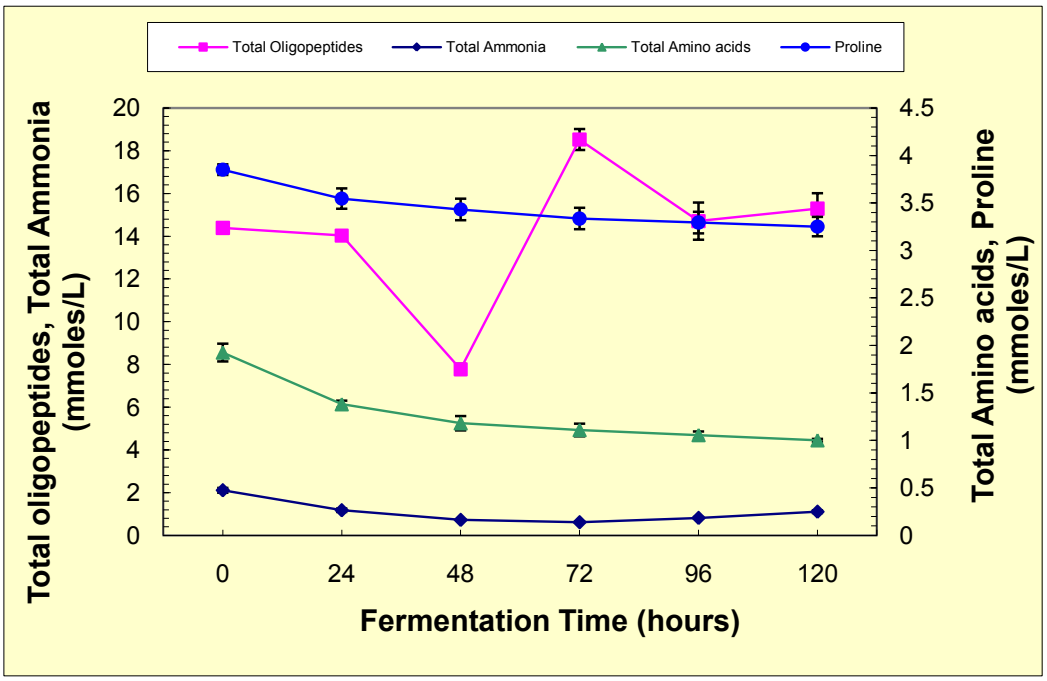


Figure 88: Total nitrogen fermentation absorption profile for the 12°Plato ale shake flask fermentations conducted with the ale strain No 70. The illustrated data are the mean values of three fermentations ± S.D.

During the lager fermentations, peptide removal from wort started very rapidly, simultaneous with the start of amino acid consumption, during the first 19h of fermentation (**Figure 89**). The wort oligopeptide concentration then began increasing after this point until 24h into the fermentation. Between 24 and 67h of fermentation, oligopeptide levels commenced to be used gradually by the yeast cells again. Thereafter, small peptides started to accumulate for a second time in the fermenting wort, until the end of fermentation. The residual oligopeptides that were detected in the fermented wort were found to have reached a concentration even higher than the initial level of the unfermented wort.

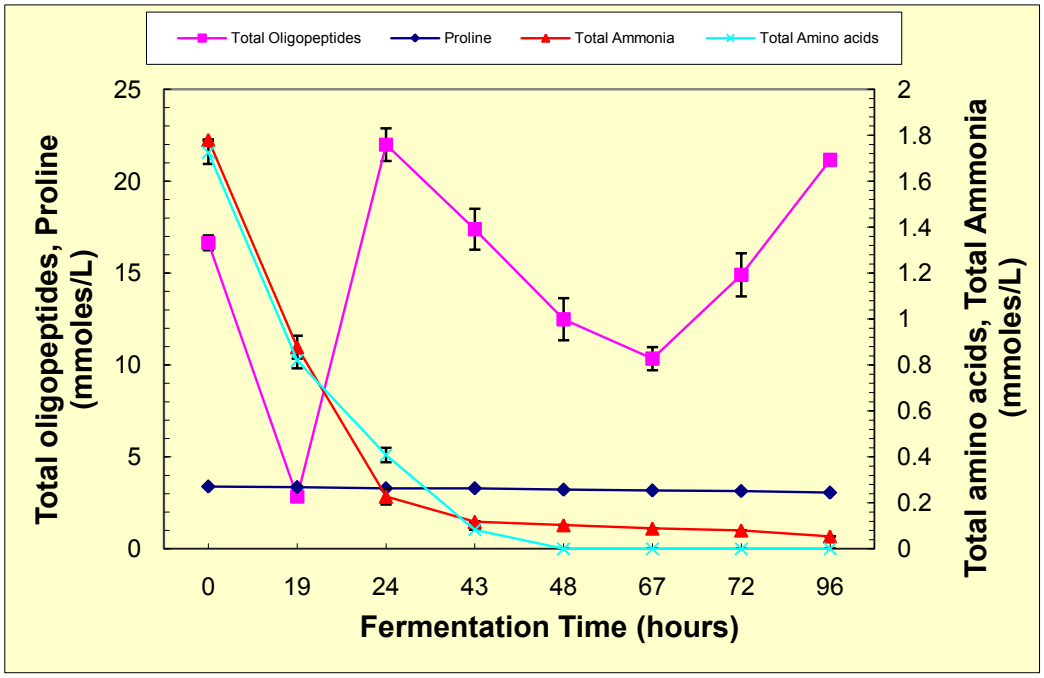


Figure 89: Total nitrogen fermentation absorption profile for the SC3 lager static fermentations. The illustrated data are the mean values of three fermentations ± S.D

A similar oligopeptide assimilation pattern was also observed for the static ale fermentations (**Figure 90**). In more detail, oligopeptide consumption also started within the first 20h of fermentation and at the same time as the beginning of the amino acid and ammonium ion absorption. For the next four hours of the experiment, oligopeptide levels increased significantly. In continuance, between 24 and 44h of yeast incubation, it was observed that small wort peptides started to be taken up once more. During the next four hours of the fermentation, the levels of small peptides increased from 9mmol/L to 19mmol/L. Then, between 48 and 72h of fermentation, the oligopeptide content underwent a very sharp decline, it being an index of oligopeptide utilization by the yeast cells. Finally, during the last 24h of the experiment, the concentration of single peptides increased. In both lager and ale fermentations, all the wort free amino acids were consumed by the yeast within the first 48h of fermentation as expected from previous experiments conducted by Jones and Pierce (1964). However, such an effect did not take place during the ale shake flask fermentations since complete uptake of wort amino acids never occurred. In all fermentations conducted, ammonia and proline utilization was incomplete as occurred during the first time when these fermentations were carried out by using the same yeast strains for pitching.

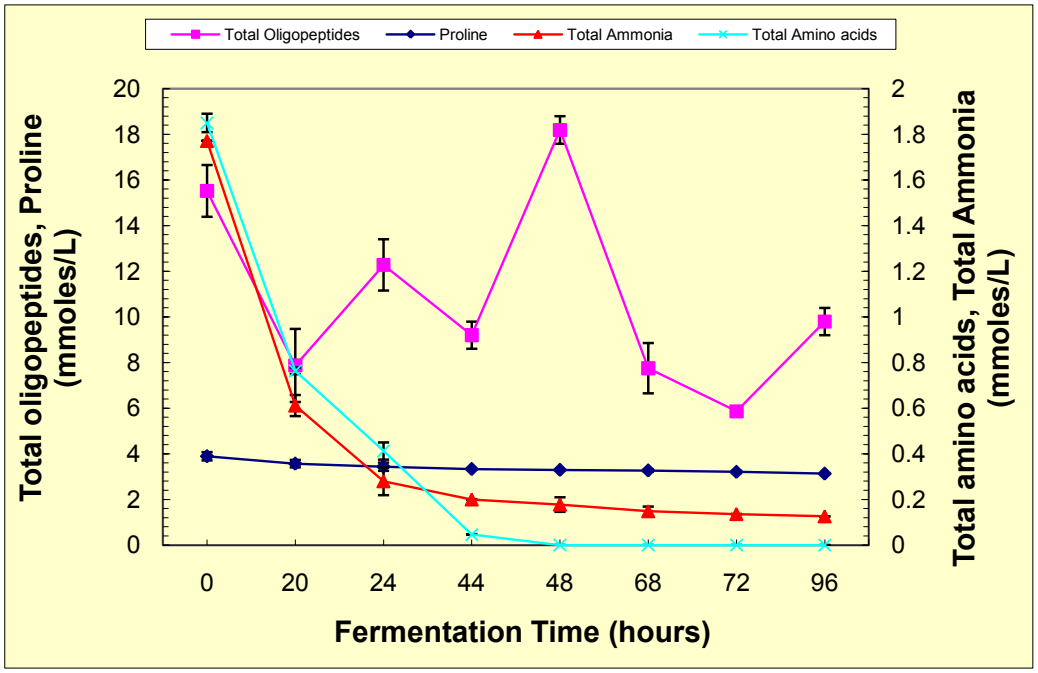


Figure 90: Total nitrogen fermentation absorption profile for the SC8 ale static fermentations. The illustrated data are the mean values of three fermentations ± S.D.

Reasons for the fluctuations that were observed to the oligopeptide levels in wort during the fermentations that were studied can be considered employing three hypotheses. The first hypothesis was that these increases in the small peptide wort concentration may have been induced by the low cell viability and consequently due to the cell lysis and release of intracellular yeast proteolytic enzymes into the fermentation liquor. The released proteolytic enzymes due to cell lysis could be responsible for the degradation of wort proteins and polypeptides into smaller peptides and thus more yeast assimilable nitrogen sources were produced. Regardless the fact that cell viability was high (90%) even at the end of the fermentation and also by measuring the extracellular ATP as an index of cell autolysis, it was found that the ATP metabolic behaviour was irrelevant to the oligopeptide absorption pattern, the cell lysis factor and the consequential release of yeast proteases in wort should be taken under consideration as a possibility to explain such an effect. The second assumption that was made in order to clarify such a phenomenon was that yeast cells excrete the small peptides that have already been taken up back into the fermenting wort and then they absorb them again. However, this theory did not seem to be applicable when the FAN content

measured and no increase in its concentration was detected throughout the duration of the experiments. Finally, the last suggestion, that could provide a coherent explanation, was the excretion/secretion of yeast proteases into the fermentation environment that could break down larger peptides and proteins into smaller peptides in order to supply the proliferating yeast cells with available nitrogenous nutrients. With the aim of further investigating the cell lysis-protease release and the protease excretion/secretion hypotheses, the overall extracellular yeast protease activity was determined. **Figures 91** and **92** show the activity of the extracellular general protease during the course of ale shake flask and lager and ale static fermentations. During the ale fermentations conducted in shake flasks, the activity of extracellular overall proteases increased for the first 24h and then a significant decrease between 24 and 48h of fermentation was noted. Following this protease activity decline, another increase occurred for the next 24h of yeast incubation. Thereafter, the extracellular protease activity showed a slight reduction after 72h of fermentation and finally during the final day of the experiment, protease activity seemed to have increased again until the completion of the fermentation.

During the lager fermentations (**Figure 92**), protease activity began to increase gradually after 24h until 48h of fermentation. Between 48 and 67h of fermentation, the protease activity remained constant and then after 72h, it started to increase again until the end of fermentation, when most individual amino acid levels had been depleted. The proteinase activity during the ale fermentations displayed a similar pattern. The only noticeable difference was that the proteinase activity decreased between 48 and 72h of fermentation, and then it started to increase again until the fermentation was complete. This was probably due to break down of larger into smaller peptides, which were used by the yeast cells after all the available other nitrogen sources had been exhausted. This suggests that yeast fermentative activity does not cease when free amino nitrogen is depleted, based on the observation that yeast proteases continue to be either released from dead cells or be secreted/excreted, from viable cells, digesting polypeptides into oligopeptides thus making more utilizable nitrogenous materials available to the yeast culture.

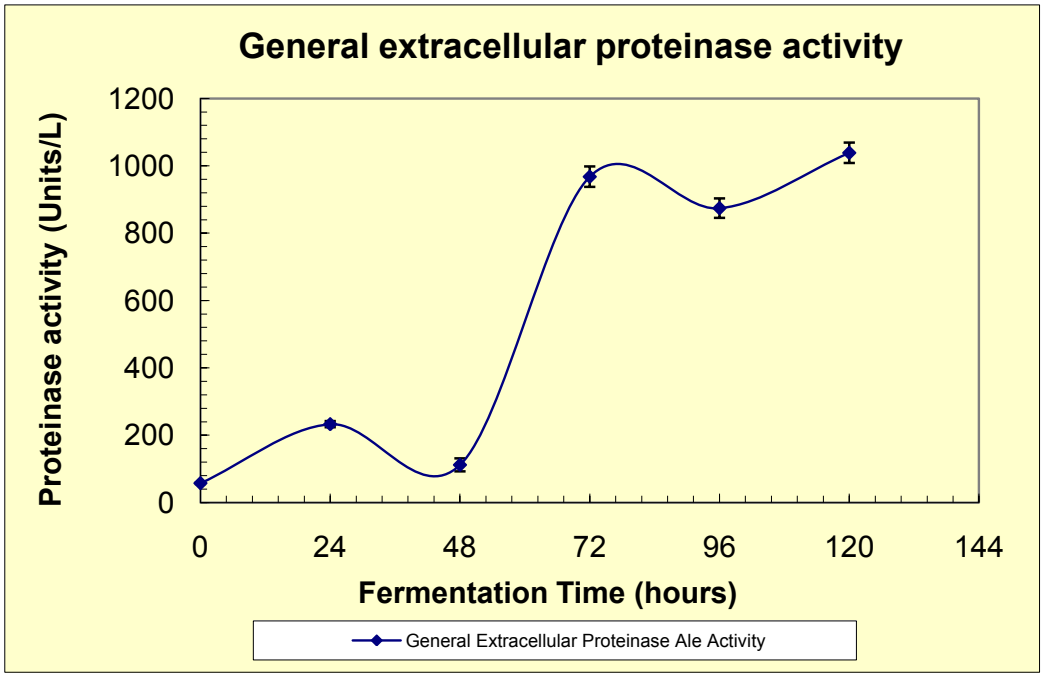


Figure 91: Extracellular proteinase activity for the 12°P shake flask fermentations conducted with the ale yeast strain No 70. The illustrated data are the mean values of three fermentations ± S.D.

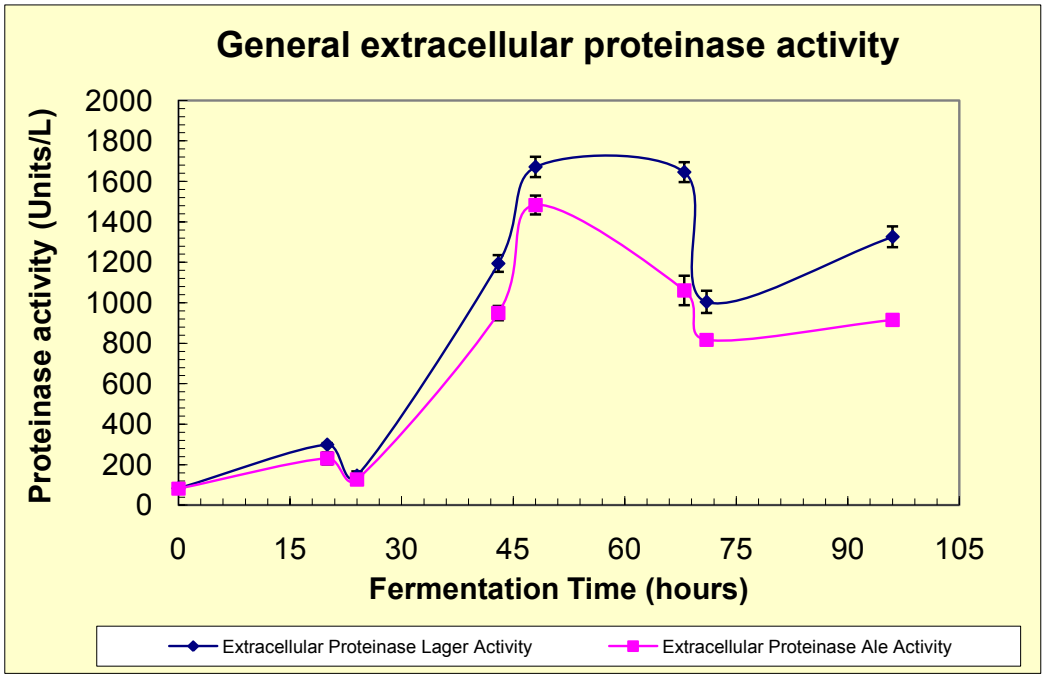


Figure 92: Extracellular proteinase activity for the static lager and ale fermentations conducted with yeast strains SC3 and SC8, respectively. The illustrated data are the mean values of three fermentations ± S.D.

This study has shown that both lager and ale yeast strains, regardless of the experimental conditions, can simultaneously use amino acids and small peptides as sources of assimilable nitrogen. In addition, it is believed that extracellular proteolytic enzymes are responsible for the degradation of larger wort peptides into smaller ones with result yeast cells to be provided with more available assimilable nitrogen sources. As already discussed, the detection of extracellular proteases in wort may have been induced due their excretion by either viable yeast cells under stress conditions (continuous agitation, high percentage of fermentable sugars) or due to their release in wort by dead and autolyzed cells.

3.6 Mashing trials for 28 different malt types

Figure 93 illustrates the hot water extract (HWE) of the 28 malt samples analyzed. The HWE is one of the important key quality malt attributes, which determines the malting performance of barley and the degree of grain modification. The blue colour represents the hot water extract (as is) and the purple colour the dry hot water extract (L^o/Kg) measured after mashing all malt types at 65^{o}C. As is shown in **Figure 93**, the malt varieties with the highest HWE are the following: GR59 (317 L^o/Kg), G019 (317 L^o/Kg), N010 (317 L^o/Kg), N011 (317 L^o/Kg), GR77 (318 L^o/Kg) and K13/52 (318 L^o/Kg). On the other hand, the malt samples with the lowest HWE values are: G016 (307 L^o/Kg), K19/49 (308 L^o/Kg), G365 (309 L^o/Kg), N019 (309 L^o/Kg) and GR32 (310 L^o/Kg). In addition, **Figure 94** depicts the yeast assimilable nitrogen content of each malt variety in the grain and the free amino nitrogen concentration that was formed after the malted barleys were mashed at 65^{o}C in order to further break down the larger nitrogenous materials.

As it can be seen in **Figure 94**, in the malt varieties N211, N010, K19/49, K12/32, K20/40, N259, G019, GR1141, G016 and G365, the 94% of their total free amino nitrogen concentration was already contained in the malt form and the rest of the content was produced as an effect of mashing. When the next 18 malt types were examined, it was realized that approximately 80% of their final free amino nitrogen concentration was a product of the protein endosperm degradation during malting, while the remainder was formed when the proteolytic enzymes were reactivated after mashing of the samples. With these observations in mind, it can be said that 88% of the total yeast utilizable nitrogen is produced during malting and the rest is produced during mashing. Such an observation obtained during this study contrasts with published results (Lie, 1973; Taylor and Boyd, 1986), which support the position that 70% of free amino nitrogen originates during malting 30% derives during the mashing stage. Additionally, another significant observation from these series of mashing trials is that there is no correlation between the HWE value and the free amino nitrogen content of the malted barley, regardless the fact that HWE is an index of grain modification. Enari (1974) reported that the modification of the malt does not greatly influence the amino acid composition of wort. For instance, the HWE values of the malt varieties K13/52 and GR77 are the highest detected (318 L^o/Kg), whereas their percentage of free amino nitrogen content was found to be 83% and 86%, respectively. On the other hand, the malt varieties K19/49, G016 and G365 had the lowest HWE contents analyzed (308, 307 and 309 L^o/Kg, respectively), but their free amino nitrogen content was

of the highest recorded (up to 96% FAN found in malted grains). In the next plots, the malt varieties are numbered from 1 to 28 (**Table 24**).

Table 24: The 28 different malt varieties used for mashing

Malt Variety	Number
GR32	1
GR931	2
N011	3
GR875	4
GR77	5
N019	6
GR913	7
N211	8
N010	9
K19/49	10
K12/32	11
K18/51	12
K20/40	13
N259	14
N197	15
N018	16
K21/34	17
K13/52	18
G019	19
GR1141	20
D164	21
GR59	22
N258	23
G348	24
GR90	25
G016	26
C163	27
G365	28

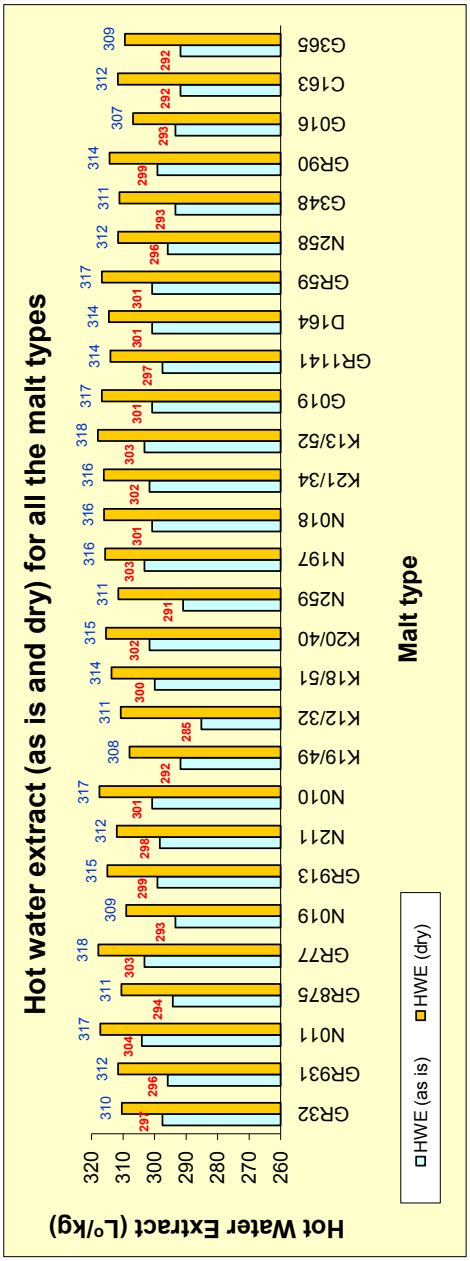


Figure 93: The hot water extract (HWE) of all the 28 malt varieties tested. Columns in blue represent the HWE (as is), whereas in purple, the dry HWE.

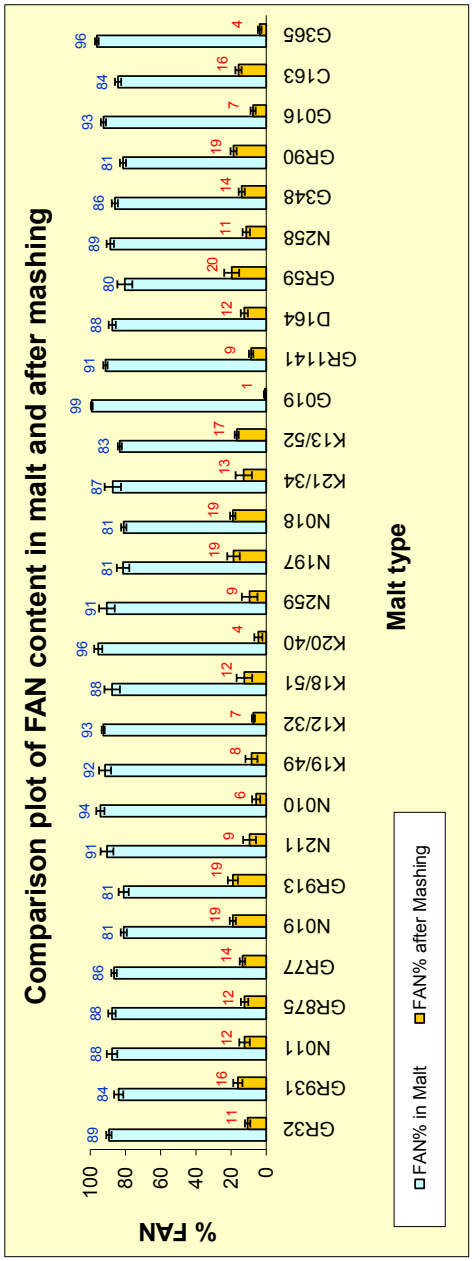


Figure 94: The FAN content of all the 28 malt varieties tested before and after mashing. The illustrated FAN percentages are the mean values of three FAN ninhydrin measurements conducted for each malt type ±S.D.

3.6.1 Amino acid spectrum of all the malt types

Figures 95 and **96** summarize the overall amino acid spectrum determined for 28 different types of malt in the grain (4°C) and in the wort (65°C). The available malt (in blue) and wort (in purple) amino acids have been presented in groups and not individually, according to their rate of absorption, for simplicity purposes. The mashing trials that were conducted at 4°C show the amino acid malt content since at this low mashing temperature, the proteolytic enzymes of the grain remain inactive and unable to induce a significant digestion to the larger sources of nitrogen. In addition, the measurements recorded at a temperature of 65°C present the additional amino acid percentage levels produced in wort when the proteolytic enzymes of the grain endosperm were active in order to break down large proteins and polypeptides into more assimilable nitrogen sources for yeast utilization.

As it can be seen in **Figure 97**, 63% of the Group 1 amino acids of all the malt types analyzed were already present in the malted grains and the rest were formed during the mashing step. Similarly, when the average Group 2 amino acid content of the 28 different malt varieties was calculated it was found that 71% of it was part of the unmashed malt form and 29% derived after malt samples were mashed at 65°C. Moving onto the examination that was carried out for Group 3 amino acids, it was found that 76% of their average concentration preexisted in the malted grains and the additional amino acid levels found in wort were products of mashing. As for the sole amino acid member of Group 4, proline, it was observed that 74% of its total wort content was found in the germinated malt grains and the remainder was formed after mashing of the seeds at 65°C. To conclude, based on these observations concerning the amino acid content of the malted grain and wort, it was found that 71% of the amino acids that are present in wort were a product of the endosperm degradation during barley germination and the rest of the amino acids were formed after the mashing stage. Enari (1980) reported that 50-70% of the amino acids present in wort are preformed in malt and the rest are generated during mashing.

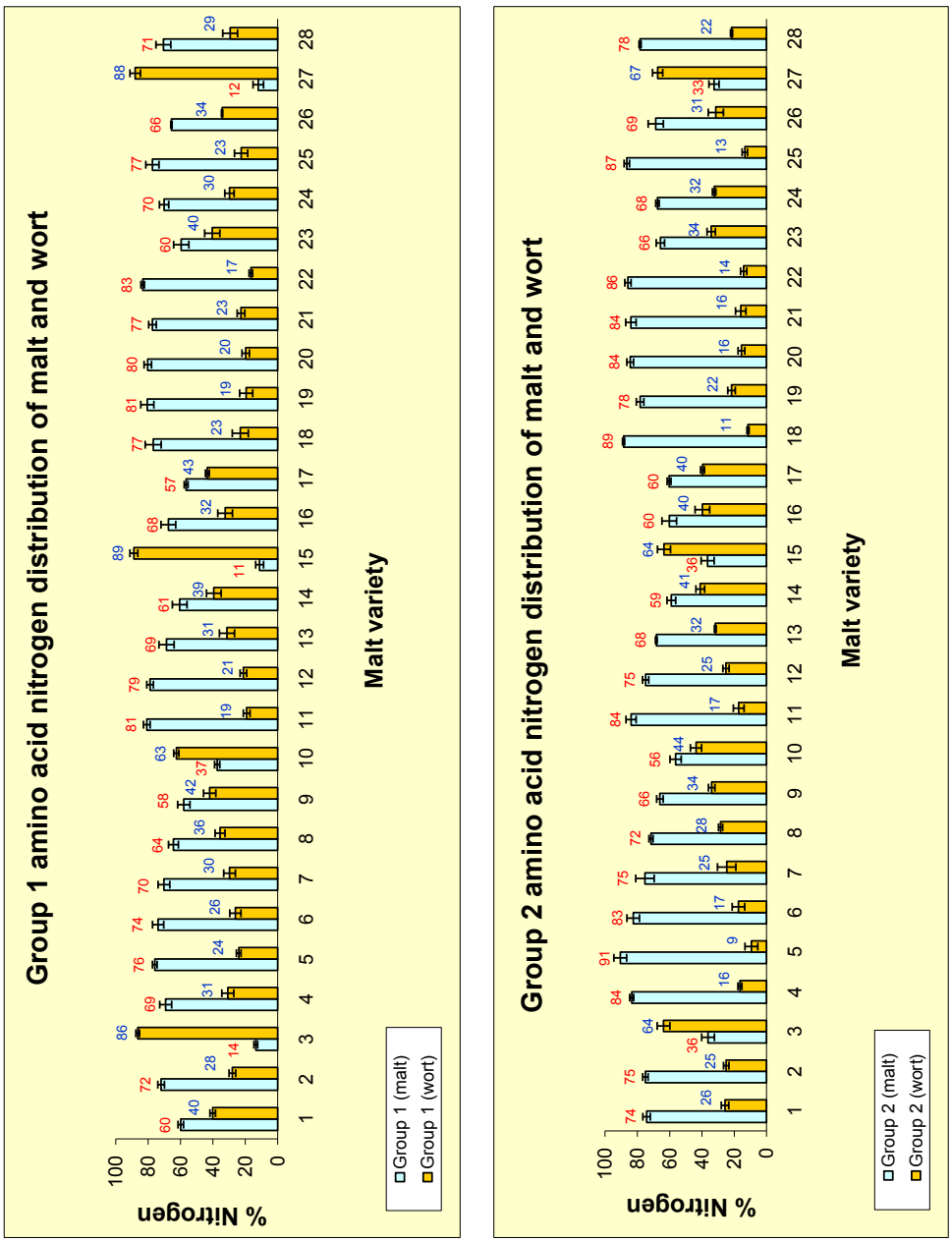


Figure 95: Groups 1 & 2 average amino acid levels examined for the 28 different malt types. The measurements taken at 4°C illustrate the malt amino acid group content while at 65°C, the amino acid group content of the wort. The illustrated nitrogen percentages present the mean values of three HPLC measurements recorded for the sum of the nitrogen components of the amino acid Groups 1 and 2 ± S.D.

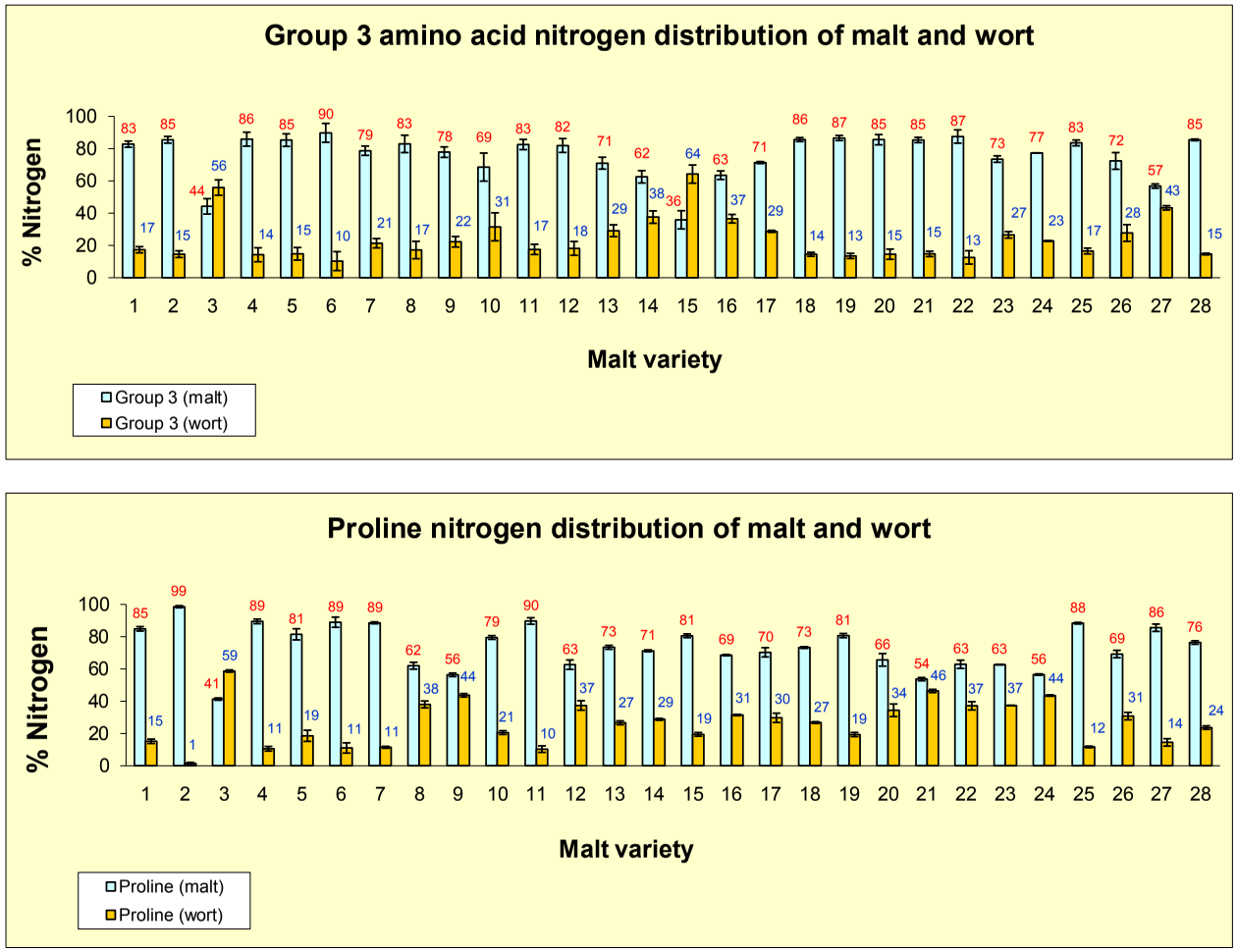


Figure 96: Groups 3 & 4 average amino acid levels examined for 28 different malt types. The measurements taken at 4°C illustrate the malt amino acid group content while at 65°C, the amino acid group content of the wort. The illustrated nitrogen percentages present the mean values of three HPLC measurements recorded for the sum of the nitrogen components of the amino acid Groups 3 and 4 ± S.D.

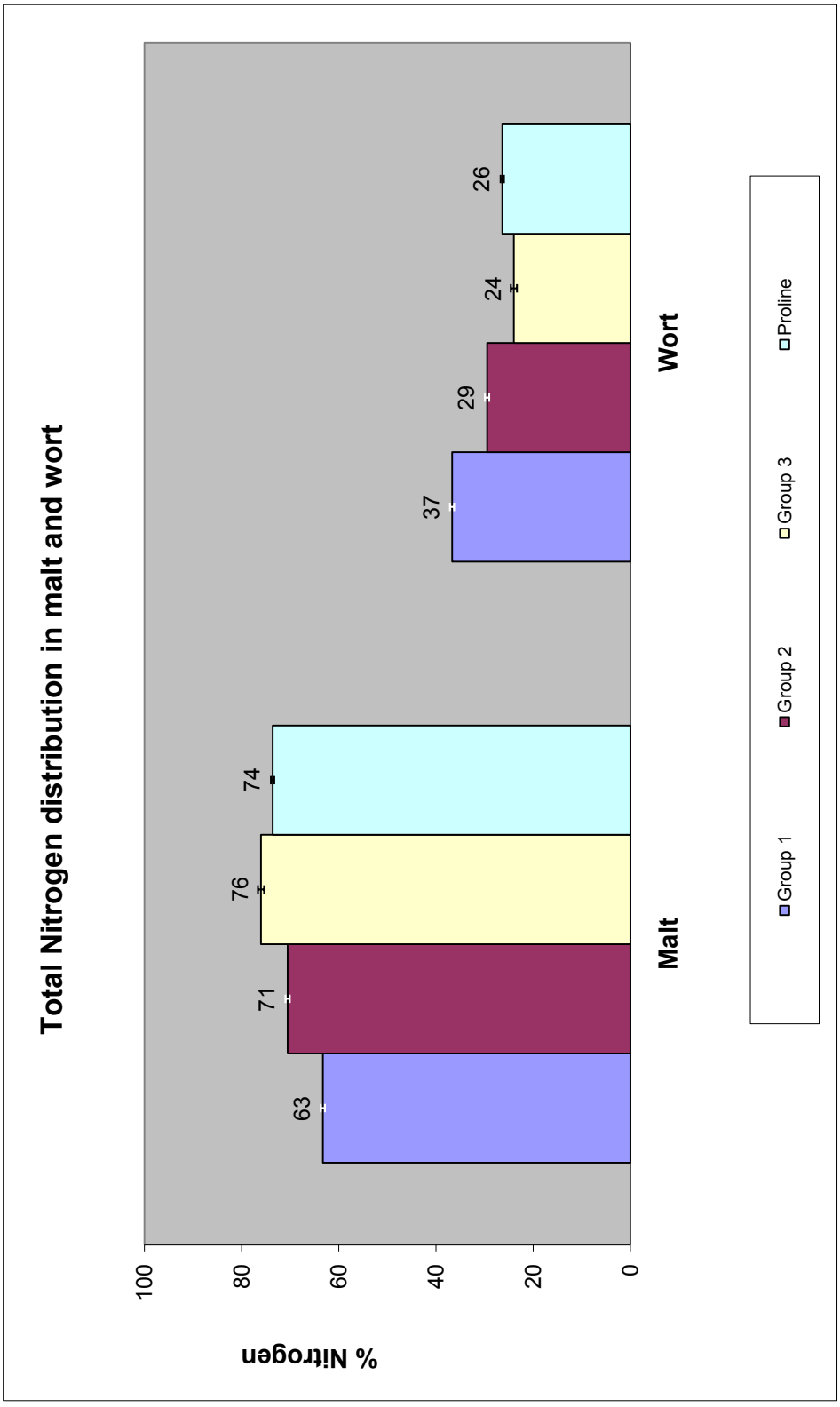


Figure 97: Average nitrogen distribution of all the malt varieties of the four amino acid groups before and after mashing. The illustrated nitrogen percentages present the mean values of 28 measurements recorded for each amino acid group ± S.D.

3.6.2 Ammonia composition of all the malt types

Figure 98 shows the ammonia levels measured for the 28 different types of malt. The results from mashing at 4°C show the ammonia malt content since at this low mashing temperature, the proteolytic enzymes of the malt remain inactive. In addition, the measurements recorded at 65°C present the ammonia levels produced in the mash when the proteolytic enzymes of the grain endosperm were activated in order to cause protein and polypeptide proteolysis.

In the malts 1, 4, 6, 8, 13, 15, 20, 27 and 28, 90% of the total ammonia level was already contained in the malted grain and the rest of it was formed during the mashing step, where large nitrogenous molecules were broken down providing further amounts of ammonium ions. Similarly, when the total ammonia content of the malt varieties 2, 3, 5, 9, 11, 16, 22 and 26 was estimated it was found that 80% of it was in the unmashed malt form and 20% obtained after mashing. With the malt types 10, 12, 14, 17, 21, 23, 24 and 25, it was found that 62% of the total ammonia concentration of these varieties was part of the malted barley. In the malt types 7 and 19, it was observed that only half of the final ammonia wort concentration was produced after mashing and the rest of it was already found in the malted grain. Finally, malt variety 18 showed that even less than half of the final ammonia wort content was the product of malt endosperm degradation and the remainder ammonia percent was formed during the mashing of the grains.

Nevertheless, if the overall mean percentage of total ammonia content is calculated for all the 28 malt types before and after mashing, it can be said that 75% of the final ammonia wort concentration used as assimilable yeast nitrogen already existed in the grain and the rest of it (25%) was formed during the process of mashing at 65°C optimum for the activation of the grain enzymes inducing protein proteolysis.

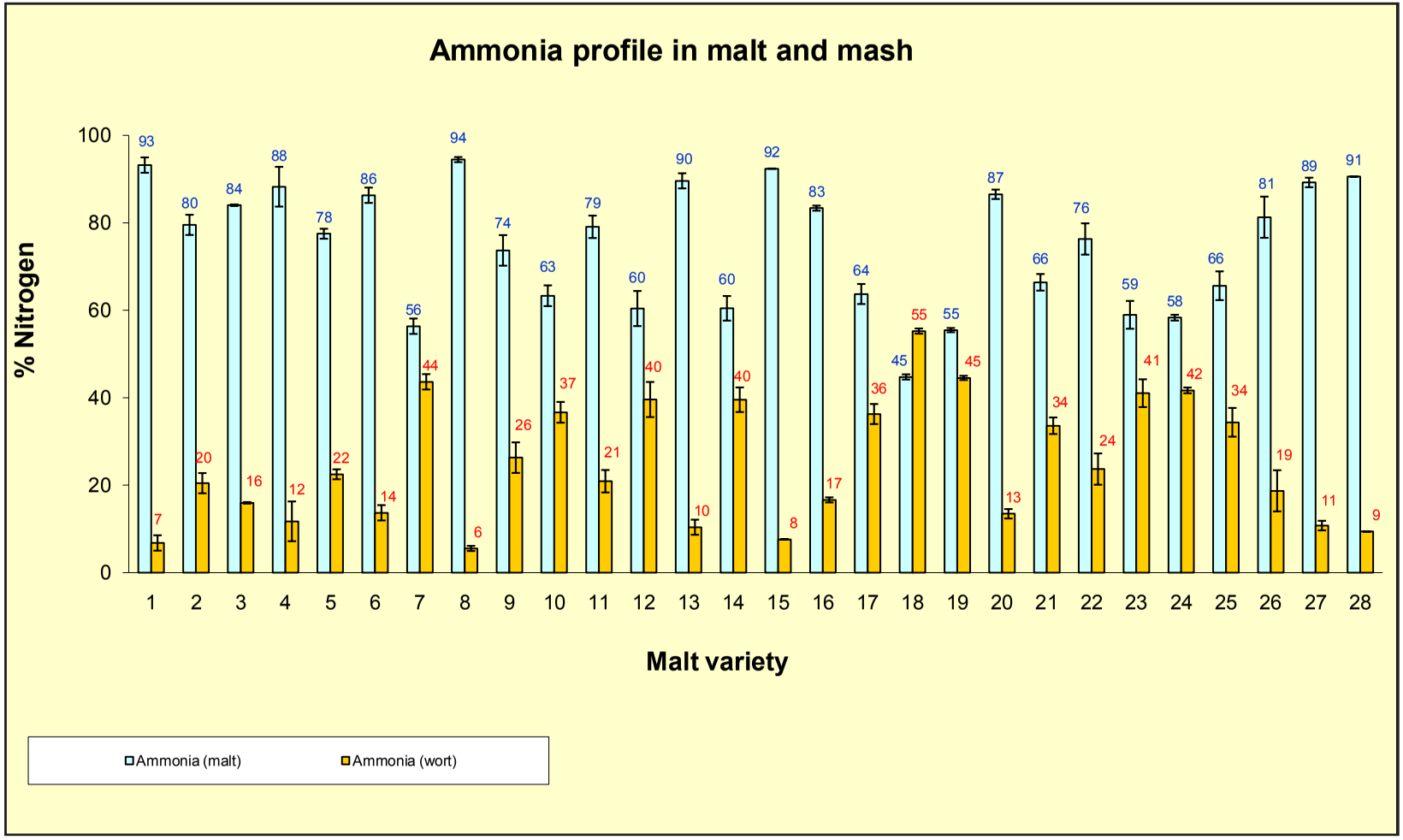


Figure 98: Ammonia levels examined for 28 different malt types. The measurements taken at 4°C illustrate the malt ammonia content while at 65°C, the ammonia content of the mash. The illustrated ammonia percentages for each malt type are the mean values of three measurements determined with the spectrophotometric ammonia detection method ± S.D.

Figure 99 illustrates the average nitrogen distribution of all the malt samples that have been tested and moreover the detailed nitrogen content of the resulting mashes. As it can be seen from the plots, the least expressed nitrogen sources in the malted grain were found to be the amino acids aspartate, glutamate, methionine and lysine, since only half of their content already existed in the malted barley and the other half, derived after the step of mashing. Then, 67% of the amino acids serine, arginine, leucine and isoleucine were found to preexist in the grain, where the remaining percentage was produced when malt samples were mashed at 65^{o}C. In continuance, approximately 73% of the amino acids asparagine, glutamine, threonine, glycine, proline, phenylalanine, histidine, tyrosine and isoleucine, including also ammonia, were found to be already present in the malted barley. Finally, only 20% of the total wort amino acid content of alanine, valine and tryptophan was observed to be a result of the mashing procedure, where the rest of it was found to have been already formed as a product of degradation of prolamins in the malted barley kernels. Such an observation means that these amino acids are the most dominant ones in the grain and mashing of the malted barley does not play a significant role in their additional formation in the final wort. On the other hand, the minor amino acids in the malted samples were found to be methionine, aspartic acid and lysine, at just 51%, 52% and 53%, respectively.

As it was shown during both lager and ale fermentations, aspartate, methionine and lysine are considered to be key nitrogen sources for a healthy fermentation, since they are removed from the wort immediately. Thus, in order that these three amino acids, crucial for the yeast nitrogen nutrition, are found in high and sufficient levels in the unfermented wort, effective mashing of the malt samples should be carried out prior to pitching.

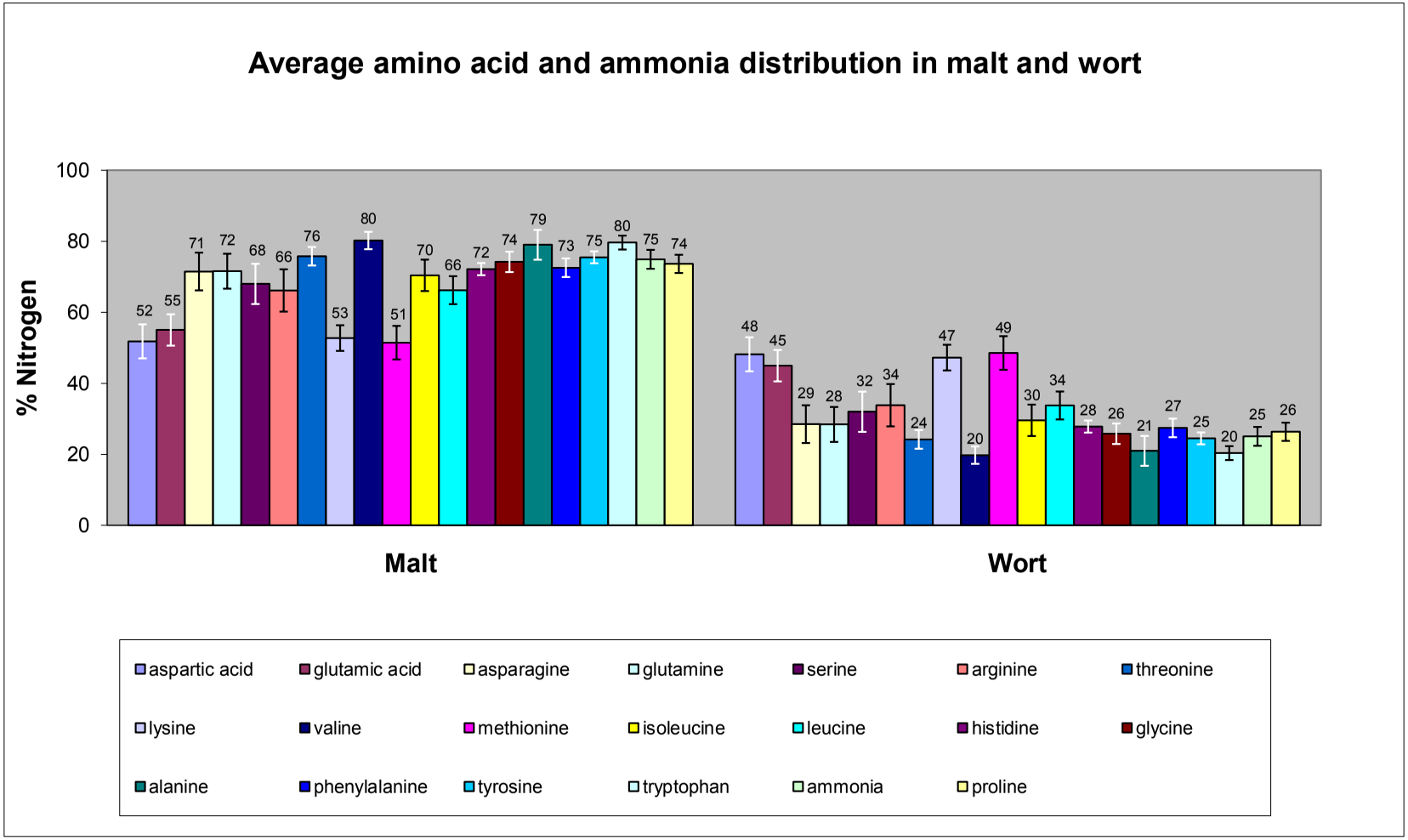


Figure 99: Average nitrogen content of all malt samples and produced mashes. The illustrated percentages depict the nitrogen proportion that each amino acid and ammonia comprises in the malted barley and in the derived wort. The nitrogen percentages are the mean values of the 28 malt type amino acid and ammonia measurements before and after mashing ± S.D.

Chapter 4: Discussion

4.1 Overall study objectives

After completion of the shake flask fermentations, it was concluded that the nitrogen sources, lysine, methione, arginine can fully support the metabolic needs of the brewing ale yeast strain No 70, for complete wort sugar attenuation. Nevertheless, a new perspective was revealed, during these fermentation conditions. The ale yeast strain (No 70) employed was found to have preferred large molecular weight nitrogen compounds in order to form more utilizable nitrogen wort constituents, rather than using the easily accessible wort single amino acids. Such yeast behaviour may have derived from the fact that continuous yeast agitation can induce an inhibitory stress effect on its metabolic performance with consequential excretion/secretion of proteolytic enzymes and the resultant nitrogen assimilable products of protein and polypeptide degradation. Alternatively, cell lysis and simultaneous liberation of yeast intracellular proteases could be also another possible explanation for the degradation of wort proteins and polypeptides.

The total nitrogen mass balance calculations that were carried out for the 12^{o}Plato all malt wort, inoculated with the ale strain No 70, shows that 73% of the yeast assimilable nitrogen consists of small peptides, 14% ammonium ions and the remaining 13%, by single amino acids. When however, the nitrogen distribution of the high gravity all malt and adjunct worts was analyzed, it was found that 17% of utilizable nitrogen derives from amino acids, 28.5% from ammonium ions and 54.5% from oligopeptides. Therefore, higher gravity worts do not necessarily contain higher levels of amino acids, since ammonia levels detected in 20^{o}Plato worts, were double that found in the normal gravity wort.

Unlike the poor and incomplete amino acid utilization performance observed during stirred fermentations, sufficient amino acid uptake occurred for all the static anaerobic fermentations conducted with the two lager and the two ale yeast strains. Nevertheless, it was possible, based on these results, to establish a general order of amino acid uptake for both lager and ale yeast strains, which is tabulated in **Table 26** (page 258).

According to mass balance calculations, the nitrogen distribution of the high gravity adjunct wort used for the static fermentations (15^{o}Plato + 30% VHM syrup), the

completion of the series of static fermentations was almost identical to that observed during normal gravity all malt wort (12^{o}Plato) fermentations used for shake flask trials. Furthermore, it was also shown that it may be possible for viable, healthy yeast cells, just hours after inoculation into wort, to commence producing additional assimilable nitrogen components by excreting/secreting proteases into the fermentation medium that will hydrolyze the peptide bonds that hold together the building blocks of proteins and other larger peptides. Additionally, the detection of proteases in the fermentation medium just hours after pitching, is possible to have been induced due to their release into wort by autolyzed cells. The theory that yeast commences to "search" for alternative nitrogen sources and consequently excretes exopeptidases only after all the available wort FAN reservoirs are exhausted, was partially confirmed, since the presence of exopeptidases in the fermenting wort may have been the result of cell autolysis.

During wort supplementation, it was also observed that increasing the concentration of wort supplements above a certain concentration had little or no positive effect on the fermentation profile. This means that individual wort amino acid levels may reach a saturation point above which there is no further positive effect on yeast performance and metabolism. Lysine can be considered to be the main "key amino acid" for stimulating a faster fermentation rate with consequential effects on beer quality and stability. Alternatively, when both methionine and ammonium ions were used as single wort nitrogen supplements, reduced suspended yeast cell numbers, lower final yeast crops and prolonged fermentation times were observed, compared to the control fermentations. The only amino acids that were statistically analyzed and found that their supplementation decreased the fermentation rate and also inhibited the yeast fermentative performance were glutamine and alanine.

In addition, it was observed that the amino acids valine, leucine, histidine and lysine had a significant effect on the production of diacetyl. In more detail, increases in the concentration of the amino acids histidine and lysine in wort led to an increase in beer diacetyl levels. On the other hand, increases in the wort levels of valine and leucine led to reduction of the diacetyl level found in beer. Furthermore, the final pentanedione concentration in beer is strongly affected by an increase in isoleucine and valine wort levels, which leads to reduction of pentanedione concentration in the fermented wort. Finally, none of the spectrum of wort nitrogenous compounds was found to significantly affect the formation of acetylaldehyde in fermented wort.

Additionally, the optimal nitrogen wort concentration was statistically calculated for the lager yeast strain SC3, with the intention that the wort fermentable sugars would be fully attenuated in the fastest possible time and also acceptable beer quality be produced containing desired levels of total vicinal diketones and acetylaldehyde.

At the conclusion of mashing, it was shown that approximately 90% of wort free amino nitrogen is formed during barley grain germination and the remaining FAN is produced during mashing by the activation of mash proteases at 65^{o}C. The literature suggests that the FAN fraction in malt and the resulting mash is 70/30 (Lie, 1973; Taylor and Boyd, 1986) but was unconvincing, since it was shown that a more realistic ratio is 90/10 (details later). However, analytically it was found that 70% of free wort amino acids originates during barley germination, whilst the rest of the wort amino acids occurs by the activation of proteolytic enzymes during mashing. Similarly, when the ammonia wort content was analyzed before and after mashing, it was found that 75% of ammonium ions were already formed in the malted grain prior to mashing.

4.2 Shake flask fermentations

Three different wort types were pitched with the ale yeast strain No 70 during agitated aerobic fermentation experiments. In order to achieve the best possible aeration (oxygenation) of the fermentations, the flask lids used were very porous so as to maximize oxygen diffusion into the wort.

At first, by examining the fermentation profile of the three sets of stirred fermentations tested (12^{o}Plato and 20^{o}Plato all malt and 20^{o}Plato malt + 30% Glucose), no significant differences in any of the fermentation parameters measured, were detected. The highest cell growth and biomass were obtained during the 20^{o}Plato gravity wort fermentations. It was also observed that the slight increase in free amino nitrogen during the late fermentation stages was a common feature for all the sets of aerobic stirred fermentations that were conducted. Such an increase in wort FAN levels during the last hours of the experiments may be correlated with the decrease of cell viability and the release of various nitrogenous materials into the fermenting wort by the yeast.

A further important observation for all shake flask fermentations is that the yeast strain exhibited complete utilization for only the amino acids lysine and arginine, which were totally absorbed after 48h of fermentation, and then methionine, which was completely

assimilated 72h into the fermentation. The rest of the amino acids of Groups 1 and 2 showed some assimilation performance, but were not completely consumed at the end of fermentation. Furthermore, Group 3 amino acids and proline were also poorly utilized, if at all. These results were true for both normal and high gravity wort stirred fermentations. Therefore, it is believed that Group 3 amino acids and proline are not preferred by yeast cells in conditions of agitated aerobic fermentations, most likely because continuous agitation of the yeast in the fermenting medium has an inhibitory effect also known as shear stress, on its fermentation performance and viability (Stoupis *et al.* 2002 and 2003). Such an effect leads to the excretion/secretion of a wide range of yeast proteases into the fermentation medium and yeast cells, instead of using individual amino acids, prefer to break down large peptides into smaller ones in order to assure the availability of more utilizable nitrogen sources. Alternatively, the possibility of yeast autolysis and resultant release of yeast intracellular proteases into wort is another theory that should be taken under consideration in order to give a valid explanation for such an effect. These two different hypotheses could be confirmed by examining the total proteinase activity of the yeast during the normal wort gravity shake flask fermentation, illustrated in **Figure 92**. The overall proteinase activity revealed that yeast cells excreted or released after their lysis, at certain fermentation intervals, various proteases that degraded large peptides into smaller peptides, as shown in **Figure 89**. Similar findings reported in the literature (Macwilliam and Clapperton, 1969; Clapperton 1971a and 1971b; Ingledew, 1975, Dreyer *et al*. 1983; Calderbank *et al*. 1985; Moneton *et al*. 1986; Pierce, 1987; Yokota *et al*. 1993; Agu and Palmer, 1999; Patterson and Ingledew 1999; Ingledew and Patterson 1999; Dillemans *et al*. 2001; Osman *et al*. 2003; Da Cruz *et al*. 2003) regarding the simultaneous usage of both amino acids and small peptides during fermentation are discussed in greater detail, later in this section. On the other hand, Jones and Pierce (1964) also reported that with high FAN content worts, the Group A amino acids were rapidly removed from the medium whereas Groups C and D amino acids were found in high concentrations in final beers, almost intact. These results suggest that if yeast is supplied with a mixture of amino acids in excess of its requirements, it preferentially absorbs the Group A and/or B amino acids, leaving behind the Group C and/or D amino acids. Such an observation that the FAN content of all the wort types, used for the implementation of the shake fermentations, was high enough might be another explanation why Group C and D amino acids remained almost untouched.

Harris and Meritt (1961) conducted highly aerated stirred fermentations and they showed that starting the fermentations with an original wort gravity of 11^{o}Plato, asparagine, histidine, phenylalanine, serine and tryptophan were totally removed from the wort by the time the gravity had fallen to 8^{o}Plato. Though, even after the wort gravity had reached its target value (2.5^{o}Plato), Group A amino acids glutamic acid, lysine, arginine and aspartate, Group C amino acids alanine and glycine and the sole amino acid of Group D, proline, were still present. Such a difference in the absorption of Group A amino acids may be due to the fact that the yeast strain that these authors used for the completion of their fermentations was from the species *Saccharomyces pastorianus* (lager yeast) and this strain may possibly follow a different amino acid assimilation pattern than the yeast species *Saccharomyces cerevisiae*. However, Jones and Pierce (1964) showed that during their series of continuously highly aerated normal gravity laboratory fermentation experiments that particularly the amino acids lysine, arginine and aspartate were removed from the medium at the early fermentation stages followed by methionine. In addition, during the same series of fermentation experiments, Jones and Pierce (1964) reported that two unidentified ninhydrin-positive materials were excreted into the wort after 16h of fermentation and their concentration constantly increased until the completion of the fermentations. It is believed that one of the two released nitrogenous materials was glutamic acid, similar to what occurred during all the ale No 70 aerated stirred fermentations. As was shown (**Figures 23**, **26 and 29**), glutamic acid commenced to build up in the fermenting medium after 24h of fermentation until the end of the experiments. The other nitrogen compound, found to accumulate in the wort during the stirred aerobic fermentations, is believed to be ammonium ions (Jones and Pierce, 1964) since during my normal gravity shake flask fermentations, ammonia was also found to be released back into the wort after 72h fermentation until the end of the experiment.

As discussed previously, an analogous assimilation pattern to the one reported by Jones and Pierce (1964) for the amino acids lysine, arginine and methionine was observed during my shake flask experiments. However, Jones and Pierce (1969), unlike Harris and Meritt (1961), found that during their aerobic fermentations no traces of proline could be detected in fermented wort, whereas in my experiments, proline utilization during all the shake flask aerobic fermentations appeared to be non existent and obviously incomplete. The same findings of Jones and Pierce (1969) regarding aerobic proline assimilation were confirmed by Ingledew *et al*. (1987). Proline during extended wort fermentation undergoes slow but complete removal during aerobic fermentations.

Palmqvist and Ayrapaa (1969) also conducted a series of stirred lager fermentations using a high gravity (20^{o}Plato) adjunct wort containing 20% rice as adjunct. Their results, concerning the metabolic activity of wort amino acids, revealed that Group A components, serine and threonine were removed from the medium during the early logarithmic phase, within 50h after the yeast pitching. Then, five hours later, the total uptake of the Group A amino acids asparagine and glutamine was completed, followed by aspartic acid assimilation, which was consumed 70h into fermentation. Finally, arginine was reported to be completely taken up by the yeast 120h into fermentation. Of the two remaining Group A amino acids, glutamic acid and lysine, lysine levels gradually decreased until 100h of fermentation and then remained more or less the same with residual levels of this amino acid detectable in the final fermented wort. Glutamic acid exhibited a slight increase after 120h fermentation and this increase continued until the end of the fermentation. Similarly, during my fermentations, glutamic acid increased after 24h of fermentation and its concentration continuously increased until the end of fermentation. On the other hand, during the stirred aerobic fermentation experiments, by examining the Group A uptake, it was found that from this group arginine and lysine appeared to be the most readily absorbed amino acids and the most important for the yeast nitrogen metabolic activity. These two wort nitrogen compounds were absorbed very rapidly after 48h of fermentation. As for glutamic acid, yeast did not show any preference at all for this wort nitrogen source and this is confirmed by comparing its absorption pattern during all the shake flask experiments conducted (**Figures 23**, **26** and **29**), where instead of being removed from the fermentation medium it was excreted back into it. The rest of the Group A amino acids were not totally utilized by the yeast cells even after 120h (for normal and high gravity all malt wort experiments) and 144h (for high gravity adjunct wort experiments) fermentation and high concentrations of them were detected in the fermented wort.

In Group B amino acids, Palmqvist and Ayrapaa, (1969) described that only methionine from this group was efficiently utilized but its uptake was linear and slow and was completely taken up after 100h fermentation. However, the ale strain No 70 managed to absorb methionine totally from wort, 30h faster than the lager yeast strain that Palmqvist and Ayrapaa (1969) used. This probably occurred because their fermentations were conducted at 8-9^{o}C, while mine were conducted at 20^{o}C. The rest of the second group amino acids, valine, leucine, isoleucine and histidine, exhibited the same assimilation pattern as observed in my aerobic fermentations meaning that high concentrations of

these free amino nitrogen components were detected in the final beer. Palmqvist and Ayrapaa (1969) also reported in their findings that wort ammonia levels were totally utilized within 100h of fermentation. However, during my normal gravity fermentations, not only was wort ammonia not consumed completely after 3 days fermentation, but its initial levels commenced to accumulate back into the fermentation medium. In the case of the high gravity aerobic fermentation experiments, ammonium ion levels exhibited a satisfactory utilization rate, but unfortunately were not totally removed from the wort at the end of fermentation.

Phenylalanine, tryptophan, tyrosine, alanine and glycine gave exactly the same results compared to my shake flask fermentations, whereas these nitrogen wort components did not appear to play a significant role in yeast nitrogen metabolism by remaining unused during the course of the fermentations conducted.

No correlation of proline uptake with continuous aeration, initial levels of nitrogenous materials or extent of nitrogen assimilation was observed during Palmqvist and Ayrapaa (1969) experiments. Proline, as expected, by looking at the results obtained from my and Palmqvist and Ayrapaa (1969) different sets of stirred fermentations, exhibited no sign of absorption. Finally, compared to my amino acid wort analysis results, no cysteine was also detected in the unfermented wort that Palmqvist and Ayrapaa (1969) used for conducting their aerobic stirred fermentations.

The first main difference between Palmqvist and Ayrapaa's (1969) aerobic fermentations and the experiment reported here was that they, as did Harris and Meritt (1961), used *Saccharomyces carlsbergensis* (now *Saccharomyces pastorianus*) to carry out their experiments. Such a difference likely had a profound effect, the variations observed were mainly in Group A amino acid utilization, since the physiological and functional properties of a yeast species may even vary from one fermentation batch to another, between different generations (cycles) of the same yeast strain or also under different fermentation conditions (Jin *et al*. 1996). It should be also added that Jin *et al.* (1996) reported that differences in the fermentation performance between different fermentations may not be caused by the lack of any particular amino acid group, but by the overall efficiency of nitrogen utilization.

The other observed difference, which may be the key explanatory factor for the above deviations in the amino acid uptake, was probably the different fermentation conditions, such as the speed of agitation. Palmqvist and Ayrapaa (1969) adjusted their stirring speed in order to maintain the yeast in suspension whereas the shaker speed used for my

fermentations was 150rpm, since it is well known that the mass transfer of oxygen is strongly affected by the rotational speed and thus the best possible aeration effect of the fermentations wanted to be achieved. However, it is believed that a high shaker speed functioned as a limiting factor, because yeast cells in suspension were stressed due to continuous agitation. Further evidence that may support this hypothesis is that when yeast cells were examined under the light microscope after 48h incubation, they were found to have modified their shape from round to elongated rods (**Figure 100**), a situation also known as pseudohyphal state (Zaragoza and Gancedo, 2000).

A **B**

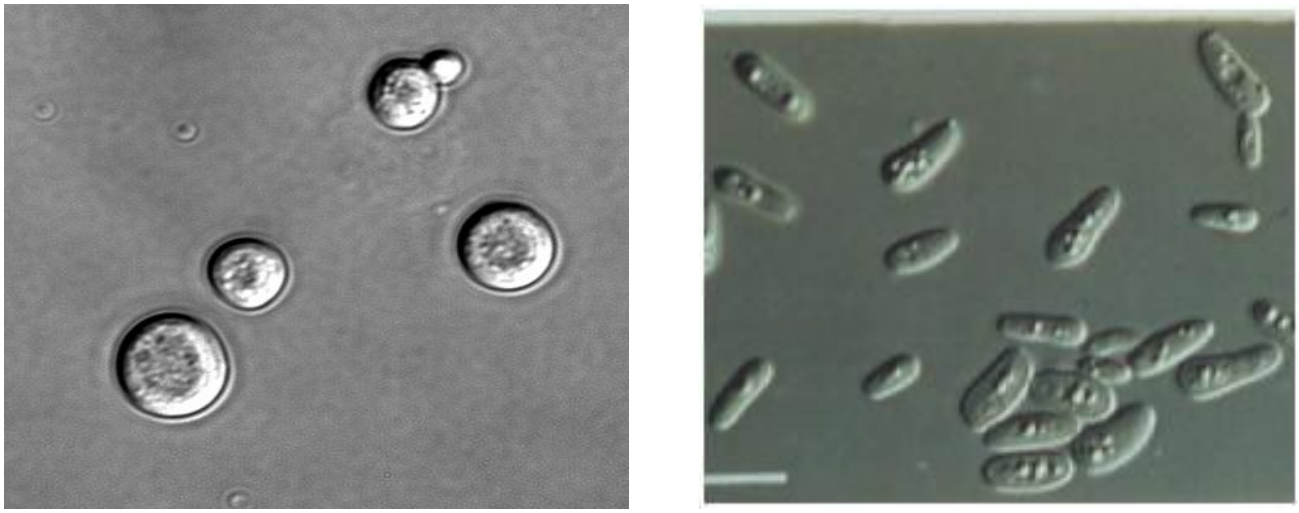


Figure 100: **A)** Normal yeast cells, **B)** Yeast cells in pseudohyphal state

Zaragoza and Gancedo (2000) reported that the pseudohyphal cell formation observed during *Saccharomyces cerevisiae* growth is a physiological yeast response not only due to nitrogen deprivation, but also to a stressful environment, where a variety of factors such as mechanical shear stress can also trigger pseudohyphal growth and invasiveness. Thus, the shear stressed cells might explain the fact that yeast cells started the excretion/secretion of proteolytic enzymes into the external environment in order to hydrolyze large peptides into smaller ones to produce more available nitrogen sources for yeast utilization (**Figures 88 and 91**). However, cell autolysis might have been the reason of the release of proteolytic enzymes in the fermentation medium. Stoupis *et al.* (2002) also reported that the greater the rotational speed of the mechanically agitated yeast slurry before fermentation pitching, the greater the total protease concentration

produced in the slurry. The same authors later showed (Stoupis *et al.* 2003) that increase of extracellular protease activity levels and decrease in foam stability in mechanically agitated yeast slurry supernatants showed satisfactory correlations with shear rate and shear stress during continuous agitation. The decrease of foam stability in the final beer was attributed to the increased levels of protease activity during fermentation resulting in hydrolysis of foam-active polypeptides due to the agitated yeast slurry used for pitching (Stoupis *et al.* 2003).

Finally, by analyzing the results derived by the total nitrogen mass balances calculated for all the wort types used for the conduct of all the shake flask fermentations, the following table (**Table 25**) can be constructed. **Table 25** also includes the estimated percentages of oligopeptides that are believed to be a fraction of the FAN content of the three different wort types, since the ninhydrin FAN identification method also detects small peptides.

Table 25: The analytical components of the FAN content of the three different worts used for the shake flask fermentations

Wort Type	Group A	Group B	Group C	Ammonia	Proline	Peptides
12^{o}Plato	3.3%	2.9%	3.6%	13.7%	3%	73.5%
20^{o}Plato	4%	3.8%	5.4%	25.6%	3.6%	57.6%
20^{o}Plato + 30% Glucose	3.8%	4.1%	5.3%	31.6%	3.8%	51.4%

4.3 Static fermentations

As shown in the Result Sections (***3.2.1*** and ***3.2.2***), yeast growth during all the static fermentations was affected by wort FAN. The amount of wort FAN required by yeast under normal brewery fermentations is directly proportional to yeast growth (Pugh *et al.* 1997). Thus, wort should contain sufficient FAN levels to support healthy yeast proliferation, otherwise detrimental effects on the yeast fermentation performance are induced, resulting in sluggish and incomplete (stuck) fermentations (Pugh *et al.* 1997). However, it was realized that the sluggish fermentative performance observed during the ale yeast fermentations pitched with the yeast strain SC5 was not induced due to free amino nitrogen deficiency, but because of other reasons discussed later in this section. Pugh *et al.* (1997) reported that there is not a specific wort FAN composition that can be universally suggested to brewers, as FAN requirements depend on the amount of yeast growth required to obtain the beer flavour and stability desired by the brewer, under a given set of fermentation parameters, such as wort gravity, fermentation temperature and the duration of primary and secondary fermentation.

All the static anaerobic fermentations, that were carried out with the two lager and two ale yeast strains, exhibited adequate amino acid utilization and unlike the aerobic stirred fermentations, their nitrogen assimilation was found to be complete with the exception of proline and ammonia. It should be also added that no cysteine or cysteic acid were ever detected in any of the samples analyzed. It is believed that the absence of this particular amino acid from the wort is due to its conversion into wort flavour (volatiles) and/or colour (melanoidins) compounds via the non-enzymatic reaction, also known as the Maillard reaction. The Maillard reaction predominantly occurs during wort production due to the high temperatures used during mashing and wort boiling. The Maillard reaction is a chemical reaction between an amino acid and a reducing sugar and it requires heat to be activated. The carbonyl group of the sugar reacts with the nucleophilic amino group of the amino acid and forms a variety of molecules responsible for a range of odors and flavors. Coghe *et al*. (2005) reported that cysteine levels in the Pilsner wort type used during their experiments were negligible. In addition, they reported that levels of the wort single amino acids decrease with the colour of wort, in other words, the darker the malt after roasting, the lower the levels of the single wort amino acids. Coghe *et al.* (2006) also observed that the higher the roasting time and temperature, the lower the wort amino acid content, due to the Maillard reaction between the fermentable sugars and

single malt amino acids. Therefore, it is believed that the concentration of cysteine is low in the malted barley and it is converted partially into sulphur containing flavour or colour compounds during mashing and wort boiling due to the non-enzymatic browning.

The nitrogen assimilation behavior of the two ale and the two lager yeast strains and their repitched fermentations can be taken under consideration so that a novel classification amino acid absorption pattern may be established (**Table 26**).

Table 26: Classification of wort amino acids and NH_3 according to their consumption rate by two ale and two lager yeast strains and their repitched fermentation data

Group A **Fast uptake**	**Group B** **Intermediate Uptake**	**Group C** **Slow Uptake**	**Group D** **Little or No Uptake**
Glutamic acid Aspartic acid Asparagine Glutamine Serine Threonine Lysine Arginine **Methionine**[2] **Isoleucine**[2] **Leucine**[2]	Valine Histidine **Tryptophan**[3] **Tyrosine**[3] **Phenylalanine**[3]	Alanine Glycine Ammonia*****	Proline*****

Key (*): Incomplete utilization.
Key ([2]): Initially belonged to Group B, according to Jones and Pierce (1964)
Key ([3]): Initially belonged to Group C, according to Jones and Pierce (1964)

By examining **Table 26**, significant observations can be obtained regarding the assimilation of various individual amino acids. Methionine, for instance which was classified by Jones and Pierce (1964) as a Group B constituent, was found during all the static anaerobic first series and repitched fermentations to exhibit very fast utilization and was completely removed from the wort during the first 18-24h of yeast incubation. It was also found to be one of the three amino acids preferred by yeast during the shake flask fermentations. Such an effect may reveal the need yeast has for sulphur, since as it has already been mentioned, that no cysteine or cysteic acid has ever been detected in

wort, when the whole spectrum of amino acids were analyzed. Jones and Pierce (1969) also consistently observed complete utilization of methionine under their fermentation conditions, but they classified it in the Group with amino acids performing intermediate absorption (Group B). In addition, Jin *et al.* (1996) also studied the absorption behaviour of methionine, but this time they classified it as a Group C amino acid. Jin *et al.* (1996) tried to present a logical explanation for such a deviation of methionine uptake from the initial Jones and Pierce (1964) assimilation pattern. They suggested that methionine levels in the wort they used averaged 3.8mg/L, while in Jones and Pierce's (1964) samples, the average methionine wort concentration was 2.5mg/L. Hence, they concluded that this difference in the initial levels of methionine in wort could provide a valid explanation about the different utilization pattern and classification between their methionine uptake rates and that observed during Jones and Pierce (1964) fermentations. Nevertheless, such an explanation does not seem to be valid since the initial average methionine concentration in the wort type used during my experiments was 3mg/L and according to Jin *et al.* (1996) suggestions about the initial methionine levels and classification, this amino acid should have exhibited an intermediate uptake. Even during the supplemented fermentations with two and five times the natural wort levels of methionine, yeast cells managed to absorb it simultaneously along with Group A amino acids, within 48h of fermentation, as shown in **Figure 65**. Therefore, with all these observations in mind, it was concluded that methionine should be reclassified, since it seems to belong in Group A, with the other amino acids that undergo fast absorption by yeast cells. The same observations were also valid for the Group B amino acids leucine and isoleucine. These two amino acids were found to have been totally removed from the wort during most of the fermentations conducted, along with the rest of the Group A amino acids, within the first 24h of fermentation. Thus, it is believed that these two wort nitrogen components should also be reclassified as Group A amino acids.

In addition, the amino acids tryptophan, tyrosine and phenylalanine, based on the Jones and Pierce (1964) amino acid consumption model, belonged to Group C. Nevertheless, it was shown during my static fermentations that these amino acids exhibited intermediate absorption rather than slow and disappeared from the wort within 42-45h after the start of fermentation. However, Izquierdo-Pulido *et al.* (2000) have a different view about tyrosine since during static fermentations they found very high levels of tyrosine to be unused in final beer. The findings of these researchers suggested that tyrosine exhibits the same utilization behavior as proline and that it should belong in Group D. Such an

absorption pattern result for this amino acid was only observed during my aerobic stirred fermentations, but this was a common phenomenon for all the amino acids of Group C, based on the Jones and Pierce (1964) model. Furthermore, from the data obtained for the amino acids glycine and alanine, it can be said that only these two amino acids exclusively belong to the Group C as they exhibited slow consumption during all the fermentations carried out. The rest of the amino acids of the Groups A, B and C seemed to have followed the same uptake rates as reported by Jones and Pierce (1964).

As already discussed, the only simple wort nitrogen components that did not undergo complete absorption were proline and ammonia. Brewer's yeast is able to assimilate proline under aerobic conditions as a result of the activation of the mitochondrial yeast enzyme proline oxidase, which is responsible for the oxidation of proline to Δ^1-pyrroline-5-carboxylate (P5C) and then P5C is converted to glutamate by the action of P5C dehydrogenase (Ingledew *et al*. 1987). However, ammonia appeared to have exhibited up to 92-96% utilization in most of the fermentation experiments. Jones *et al.* (1965) suggested that during static fermentations, yeast cells began absorbing ammonium ions only after most of the wort amino acids had been depleted. However, these findings are not in agreement with my results, where it was found that ammonia in wort was taken up by yeast at the same time that other amino acids were absorbed from the medium and its utilization was constant until the first 44-48h of fermentation. Thereafter, ammonia levels remained more or less the same until the end of fermentation. Ingledew (1975) reported that amino acids and small quantities of inorganic nitrogen (meaning ammonium ions) are totally utilized within the first 40h of fermentation. These data confirm my observations about free amino nitrogen utilization that all amino acids, with the exception of proline, are removed from the wort within the first 44-48h of fermentations (Ingledew, 1975). As for ammonia, during Ingledew's (1975) experiments, the initial levels of this particular wort nitrogen constituent were measured and found to be less than 13mg/L, whereas in the wort type used for my fermentations, the ammonia starting concentration was even higher than 30mg/L. This factor may constitute a possible reason why in my experiments wort ammonium ions were not completely used and after 48h fermentation, ammonia utilization exhibited a plateau pattern. Moreover, it can be assumed that the wort type that Ingledew (1975) used lacked sufficient levels of amino acids, so yeast utilized all the available nitrogen sources to accommodate its nitrogen nutritional needs.

Jones and Pierce (1964), during their amino acid and ammonia classification experiments, compared the absorption performance of the same ale yeast strain pitched in two different free amino nitrogen content worts. The free amino nitrogen content of the first wort tested was 154mg/L, while for the other wort type was 191mg/L. They found that all the available amino acids of the first wort type were completely utilized under the sequence pattern that they initially established, but during the fermentations conducted with wort with the higher FAN content, the same yeast strain exhibited different preferences as to which amino acids were more beneficial for its metabolic needs. High levels of Group C amino acids glycine, alanine, tyrosine, phenylalanine, tryptophan and proline were detected in the final beer. Because of these results, Jones and Pierce (1964) suggested that if yeast is provided with a mixture of amino acids in excess of its requirements, it preferentially absorbs the Group A and B acids, leaving behind those of Group C and D. However, such an observation does not seem to be valid based on my results. To be more specific, the FAN content of the wort type used for my static fermentations was estimated to be approximately 185mg/L. By gradually analyzing the amino acid and ammonia content of the fermenting wort at the end of fermentation, all the amino acids were removed from the medium within 48h of fermentation with the exception of proline and some residual levels of ammonium ions which were left unconsumed. Moreover, it was also shown that yeast during fermentation not only uses the available wort amino acids, but also tries to form additional available assimilable nitrogen sources by breaking down high molecular weight nitrogen molecules from the wort. Thus, it can be also assumed that possibly these unused amino acids were products of extracellular yeast proteolytic activity due to cell lysis or cell excretion and larger protein and peptide fragments were broken down into smaller ones. Additionally, Pickerel (1986) reported that at the end of the fermentations conducted with high FAN worts (180-190mg/L), the final FAN levels of the fermented wort were often higher than the initial levels. It was suggested that such an effect may be due to the excretion of nitrogenous substances in the medium by yeast cells as was also reported by Clapperton (1971a). However, in my view the action of extracellular proteases, which could be released after cell lysis or excreted by yeast cells, would appear to be responsible for increases in the final FAN levels and significant amounts of residual amino acids in the fermented beer.

As discussed in detail in this document, Jones and Pierce (1964) classified the wort amino acids according to their rate of utilization based on the time taken from yeast

pitching for each amino acid to achieve half of its initial concentration. Pierce (1987) also suggested that the rate of absorption of individual wort amino acids during fermentation is a constant sequence, which is almost independent of the fermentation conditions employed. However, as it was already shown during my fermentations, this classification of absorption sequence is not rigid and great variations can occur from fermentation to fermentation and by also using different yeast strains. Furthermore, it was shown that during my shake flask fermentations under continuous aeration and agitation conditions, brewing yeasts appear to have different preferences for amino acid and ammonia uptake. Moreover, the methods that these researchers used for categorizing the wort amino acids according to their removal rate, does not distinguish between those which are completely but slowly removed from the medium and those which are removed after a long lag period.

Furthermore, it was calculated that the assimilable nitrogen content of the high gravity adjunct wort used for conducting these fermentation experiments contained 3% Group A amino acids, 2.5% Group B amino acids, 3.5% Group C amino acids, 3.5% proline and approximately 14% ammonium ions. The remaining free amino nitrogen content of the wort, is believed to be composed of small peptides detected by the ninhydrin test that also play a role in yeast nitrogen metabolism.

Additionally, by comparing the fermentation profiles exhibited by the first generation of the yeast strains to the fermentation profiles of the repitched trials, it was concluded that the fermentation parameters measured exhibited almost an identical mode of change and also that the ageing factor did not play any inhibitory role on the yeast fermentation performances. The same observations were also made when final fermentations measurements such as total ABV % and total yeast crop were measured.

Nevertheless, the only problem that was encountered regarding the performance and rate of the static fermentations was during the ale yeast strain SC5 fermentations. As shown in the Results Section (**Figures 43 and 46**) during these fermentations pitched with the strain SC5, the first set of fermentations were not completed even after 162h, while similarly the repitched fermentations did not manage to achieve their target gravity even after 168h. At first, such an effect was directly associated with the rapid utilization of the wort free amino nitrogen and that after 24h fermentation limiting amounts of amino acids were available in the fermentation medium. Nevertheless, after further investigation, the total nitrogen yeast uptake was analysed and surprisingly it was found that the very poor fermentation performance of this yeast strain, was not caused by the early wort nitrogen

deficiency but by other fermentation parameters. Therefore, these fermentations were repeated in order to find the reason for such an effect. However, this time it was realized that after 15h yeast incubation, a limiting number of cells were in suspension to perform efficient attenuation of the wort fermentable sugars. The same effect was also observed during the repitched fermentations and based on visual observations, it was noticed that yeast flocculation commenced rapidly during the first 12h of fermentation and a large amount of the yeast crop had already flocculated to the bottom of the cylinders. In addition, when Scottish Courage was asked about the flocculation characteristics of this strain, they replied that SC5 was not a very flocculent yeast strain and that they never faced similar sedimentation problems when they conducted fermentations in EBC tall tubes using a similar wort type that it was used during my experiments. Consequently, we came to the conclusion that the fermentation problem originated from the geometry of the fermentation vessel used and that the 2L cylinders used did not ideally facilitate the fermentative ability of this particular yeast strain. Jin *et al.* (1996) also reported that variations in fermentation conditions may also affect the fermentation efficiency and even the presence of sufficient assimilable nitrogen does not always guarantee a good fermentation performance particularly in static conditions. Pierce (1987) reported that the general amino acid absorption pattern is not affected by the flocculent characteristics of the yeast strain. This statement totally agrees with my findings regarding the SC5 flocculation problem, where yeast cells settled rapidly ($0.45x10^7$cells/ml in suspension after 42h fermentation), but the assimilation of wort amino acids and ammonia was carried out efficiently.

4.4 Oligopeptide assimilation during stirred and static fermentations

In the Results Section **3.5**, it was shown that during both stirred and static ale and lager fermentations, yeast strains are able to utilize simultaneously wort amino acids, ammonia and small peptides. The interesting observation from these experiments was that this synchronized utilization of individual amino acids, ammonium ions and small peptides commenced from the early stages of fermentation (19-24h), when the fermentation medium contained readily available free amino nitrogen compounds. Additionally, the concentration of wort peptides with molecular weight less than 500 Daltons fluctuated at various stages of the fermentation and this aspect was carefully monitored. A valid explanation had to be developed for this effect and the stirred and static fermentations

studied for oligopeptide determination were also examined for the total activity of the extracellular proteolytic enzymes released by autolyzed yeast cells or excreted by stressed yeast cells into the fermentation medium. It was found that increases in the level of wort small peptides were correlated with the activity of the yeast produced or released after cell lysis proteases, whose main function is to hydrolyze large peptides into smaller peptides producing additional assimilable nitrogen sources. Furthermore, the fact that the oligopeptide levels during all the fermentations studied, increased at the end of the fermentation, suggests that yeast fermentative activity does not cease when free amino nitrogen is depleted and yeast proteases continue to be excreted/ secreted or relesed in the wort by lyzed cells. Consequentially, additional utilizable nitrogenous materials are produced and the progress of the fermentation continues.

Thomas *et al.* (1993 and 1996) demonstrated that *Saccharomyces cerevisiae* under conditions of high gravity brewing needs extra FAN to accomodate the higher osmotic and other stresses. This observation can also be considered as a possible explanation for the degradation of large peptides by the action of extracellular yeast proteases as occurred during high gravity wort static fermentations. Thus, it can be assumed that in order for yeast cells to overcome the osmotic stress effects of high gravity wort, they produce greater levels of free amino nitrogen irrespective of whether the wort type used has sufficient FAN levels to support a healthy fermentation. A similar effect was also observed during the 12^{o}Plato wort stirred fermentations, where the action of yeast exopeptidases resulted in an increase of small peptide levels during various stages of the fermentations. However, as was discussed in a previous section **(4.1)**, it is believed that the ale yeast strain No 70 was stressed due to continuous agitation, also known as shear stress. In contrast, damaged yeast cells may have liberated their intracellular proteolytic enzymes, following cell lysis, with a direct result the degradation of large wort proteins and peptides into more assimilable nitrogen nutrients for the remaining viable cells. The same observations about shear stress were also reported by Stoupis *et al.* (2002 and 2003). On the other hand, Maddox and Hough (1970) confirmed that leaking of proteolytic enzymes also takes place during normal gravity beer fermentation whereas yeast cells do not suffer the same degree of stress compared to the high gravity fermentations conditions.

Island *et al.* (1987), during their fermentation experiments with toxic peptides (ethionine and *m*-fluorophenylalanine containing dipeptides), detected an increase in general peptidase activity with a simultaneous decrease of 25-30% of the total medium protein

content with the result that nitrogen deprivation did not take place, suggesting again that hydrolysis of proteins takes place with the formation of smaller simple peptides to be used as alternative sources of utilizable nitrogen.

Pierce (1987) reported that during the early hours of static wort fermentations, where the need for assimilable nitrogen is very high, additional sources of FAN are supplied by small peptides, which are absorbed from the wort and serve the same role as free amino acids. Patterson and Ingledew (1999) experimented with artificial fermentation media and found that mixtures of dipeptides and amino acids induced increased growth rates, decreased lag phases, higher cell densities and shorter growth cycles. HPLC analysis of the artificial media showed that amino acids and dipeptides from the medium were utilized simultaneously and at approximately the same rate (Patterson and Ingledew, 1999). This observation indicated that peptide uptake precedes or occurs at the same time as amino acid absorption (Patterson and Ingledew, 1999). These findings agree with my oligopeptide determination results, where during the early stages of all types of fermentation studied, large amounts of small peptides were used at the same time and with the same absorption rate as the individual wort amino acids and ammonium ions.

Pierce (1987) also reported that the amino acids glycine, alanine and proline together with ammonia were excreted by the yeast into the medium, while other amino acids and small peptides were absorbed by the yeast cells. In addition, Barnes *et al.* (1998) demonstrated that during the early stages of yeast growth, nitrogen excretion is small, but it accelerates as growth proceeds and over the growth period. The equivalent of 15-20% of the nitrogen assimilated is excreted back into the medium. In addition, the same authors reported that the endogenous nitrogen content of the yeast cells rises rapidly to a maximum level, thereafter falling to a similar level as at the beginning of fermentation (Barnes *et al.* 1998).

Maddox and Hough (1970) reported that excretion of intracellular proteases into the fermentation medium under conditions of a normal gravity brewing wort (10^{o}Plato) is possible from viable yeast cells, when the medium contains proteins and polypeptides. Their results showed that leaking of proteolytic enzymes takes place during these conditions. They also reported that the extracellular proteases that the brewing yeast releases are able to hydrolyze wort proteins and polypeptides responsible for beer foam stability, leaving smaller peptides with limited and/or no foaming properties (Maddox and Hough, 1970). Hence, yeast cells by trying to sustain growth and viability, have a negative effect on the quality and final stability of the beer. This observation was also

supported by Dale and Young (1992), who reported that nitrogenous wort components of molecular weight less than 5000 Daltons act as foam negative compounds. Maddox and Hough (1970) supported this even in the presence of considerable high amounts of amino acids, the excretion of proteolytic enzymes was not inhibited. They also reported that when the same brewing yeast strain was grown in a synthetic medium, using proteins as the sole nitrogen source, proteolytic activity was observed in the growth medium outside the yeast cells. These observations suggested that excretion of proteases took place in order to break down the proteins and convert to produce more assimilable nitrogen sources for the yeast. My results confirmed their findings that even in a sufficient and rich FAN fermentation medium, the proteolytic activity of yeast proteinases is not repressed and yeast, apart from the available nitrogen wort sources, also degrades larger nitrogen molecules into smaller ones, which are used simultaneously with the wort amino acids and ammonium ions. Moneton *et al.* (1986) observed that di- and tripeptides, which contain methionine are actively transported into the cells, where an equal number of peptide residues containing glycine were not preferred by the yeast. Probably, the rapid absorption of methionine during my fermentations is correlated with the preference of the yeast to assimilate simple peptides containing methionine. These observations indicate that the amino acid composition of peptides is an important determinant in substrate specificity of the peptide permease system of the brewing yeast. Moneton *et al.* (1986) also reported that growing yeast cells in a medium with amino acids and then adding a small peptide into the medium, did not have any antagonistic effects on the absorption of the peptide and this also is in agreement with my results that peptides and amino acids can be used simultaneously by yeast cells, and is based on the existence of separate amino acid and small peptide permeases in the yeast plasma membrane.

Agu and Palmer (1999) found, during their malting and mashing studies, that barley varieties low in assimilable amounts of amino acids can still support efficient yeast performance during fermentation. These authors concluded that such an observation indicates, once again, the fact that brewing yeasts are capable of utilizing small peptides with two, three and maybe four components, in order to accommodate their nitrogen metabolic needs. Ingledew (1975) also reported that yeasts are capable of absorbing small peptides, although their growth rate is much slower when small peptides are the sole nitrogen sources. Calderbank *et al.* (1985) reported that removal of small peptides from adjunct worts was faster compared to the rate of utilization of peptides in all malt worts. Such an observation indicates, once again, that even if the wort amino acid content

is low and probably inadequate for the efficient yeast growth, yeast uses an alternative nitrogen source such as oligopeptides. They also reported that removal of peptides from both all malt and adjunct worts, occurred at the beginning of fermentation without a substantial lag phase. Their findings are in agreement with my results, where as it has been already shown, yeast cells commence to use oligopeptides at the same time as amino acid and ammonia. However, Thorne (1949) suggested that in a complex amino acid medium such as wort, yeast assimilates exogenous amino acids intact into proteins and only when the medium becomes deficient in essential amino acids then enzyme induction mechanisms are de-repressed and amino acids start to be synthesized. Thorne (1949) suggested that yeast proteinases start to function when wort becomes depleted in assimilable amino acids and then yeast commences to explore alternative sources to support its nutritional nitrogen needs. Thorne (1949) did not conduct measurements on the activity of yeast multiple excreted proteinases in order to ascertain whether amino acid utilization and polypeptide break down occurs at the same time.

Additionally, Osman *et al.* (2003), by analyzing the overall spectrum of proteins, large and small peptides in unfermented wort and in the resultant beer, they realized that yeast did not seem to affect the proteins and polypeptides, but showed great interest in the small peptides found in wort. Furthermore, Yokota *et al.* (1993) reported that the concentration of low molecular weight oligopeptides was seen to be reduced during fermentation, suggesting that brewing yeasts also have the ability to metabolize small peptides. All these observations suggest that the nutritional needs of yeast appear to be satisfied by individual amino acids and small peptides. Nevertheless, as it was shown in my results and by other publications, that brewing yeasts have the ability to degrade proteins and polypeptides so as to produce new sources of assimilable nitrogen even when the fermentation medium is rich in individual amino acids and small peptides.

Macwilliam and Clapperton (1969) compared samples of wort and beer for small peptides and revealed that a number of peptides present in wort were absent from beer whilst others present in beer did not appear to have been present in wort. They suggested that some of the small peptides and larger protein fragments detected in beer are liberated by yeast during wort fermentation. They also reported that during some static fermentations yeast excreted more peptides into the final fermented wort than it had taken up during the fermentation. Similar observations were also made in both my stirred ale and static lager fermentations, where the final oligopeptide levels detected in the fermented wort were found to have reached concentrations even higher than the initial

ones found in the unfermented wort. However, it is believed that Macwilliam and Clapperton's (1969) explanation was only partially correct since the extra levels of small peptides found during their trials may have been derived by the action of yeast proteolytic enzymes and not totally from the excretion of peptides into the fermentation medium. Ingledew (1975) also demonstrated that yeast excretes numerous nitrogenous compounds into the fermentation medium probably after active growth has ceased. Clapperton (1971a) however did not seem to be convinced by his initial observations and carried out a subsequent study and found that by removing the yeast cells every time a fermentation sample was collected, numerous small peptides were detected in the fermenting wort and in the final beer. However, further investigations by the same author led to the conclusion that certain small peptides in wort were absorbed by the yeast during fermentation, whilst others found in beer were not present in wort suggesting that they have been formed during fermentation (Clapperton, 1971a). The same researcher (Clapperton, 1971b) later reported that small peptides were formed by the yeast extracellularly probably by excreted proteolytic enzymes hydrolyzing larger peptides into more assimilable molecules. This time, my findings during the fermentation studies confirmed the validity of Clapperton's observations, where differences were found in the concentration of the small peptides detected initially in unfermented wort and then in the completely fermented wort. These observations also verify the function of yeast's proteolytic enzymes, which either were excreted by stressed cells or released in wort following cell lysis. My oligopeptide studies have shown that utilization of the wort nitrogen content during fermentation is more extensive than is indicated by the estimation of the amino acid and ammonium ions content alone in wort and beer.

Similar observations were also reported by Dreyer *et al.* (1983), firstly during their studies they believed that when wort was boiled before pitching, the malt enzymes responsible for protein proteolysis are destroyed and no further protein degradation occurs in the next stages of fermentation and maturation. However, their initial assumption was rejected because by continuing experiments with yeast pitching of the worts, they discovered that new proteolytic enzymes could be released from damaged, dead and live cells, especially under high gravity brewing conditions where yeast cells are more stressed (Dreyer *et al.* 1983). They also reported that the nature of the yeast strain affects the production of proteases and their proteolytic activity in fermenting wort and beer (Dreyer *et al.* 1983). These observations may reveal differences in cell membrane permeability from yeast strain to yeast strain. In other words, the ability of brewing

yeasts to degrade wort proteins and polypeptides is strongly affected by both the permeability of the strain's cell membrane and the degree of proteinase excretion.

Furthermore, Ingledew (1975) by conducting various studies on the role of oligopeptides in brewing, came to the conclusion that approximately 40-45% of the nitrogen deriving from proteins and large peptides is used by yeast during fermentation, which also confirms my hypothesis that proteolysis of larger peptides takes place in order for further available sources of utilizable nitrogen to be produced. Moreover, Clapperton (1971b) reported that approximately 40% of wort peptides are believed to be used during fermentation. In my experiments the percentage of small peptides used was estimated to be 37%, close to that reported by both Clapperton (1971b) and Ingledew (1975). Another valuable observation reported by Ingledew (1975) is that polypeptides are also used, as yeast elaborates proteolytic enzymes and various oligopeptides and amino acids have been identified as derivatives of yeast proteolysis, which ultimately provides additional assimilable nitrogen for the cells. The same author supports the theory that extracellular proteolysis is significant at the end of the growth phase and also he demonstrated that proteolysis of large peptides takes place at the beginning of cell growth and may be even more important. This statement agrees with my results, where during the early hours of fermentation, the level of small peptides began accumulating in the fermentation medium indicating that hydrolysis of high molecular weight nitrogenous substances was occurring just hours after pitching of the yeast strain. In general, after 44 to 48h fermentation, cell growth ceases and although limiting ammonia levels remain available for utilization in the medium, the wort appears to be deficient in utilizable nitrogen and proteolysis of large peptides and then protein fragments commences. Ingledew and Patterson (1999) found that peptide uptake during fermentation was faster and more efficient when ammonium ions were in limiting amounts in the medium.

Dillemans *et al.* (2001) reported that the supplementation of wort with a yeast peptide complex (YPC) of low molecular weight had a positive effect on yeast metabolism. YPC was able to stimulate the fermentation rate and also to improve the wort attenuation, where the final gravity of the control fermentations was found to be 3.1^{o}Plato higher (5.6 ^{o}Plato) than the supplemented ones (Dillemans *et al.* 2001). This wort peptide supplement had a beneficial effect on both lager and ale fermentations (Dillemans *et al.* 2001). It was also found that the low MW peptide supplement induced ethanol production and stabilized yeast performance during high gravity brewing fermentations through its effect on yeast sugar utilization and stress resistance (Dillemans *et al.* 2001).

YPC facilitated the uptake of glucose by yeast cells, activating the diffusion of glucose with simultaneous positive effects on yeast cellular synthesis, yeast viability and stress resistance through stimulation of mitochondrial function for the production of mitochondrial proteins, which are involved in free amino nitrogen utilization (Dillemans *et al.* 2001).

Da Cruz *et al.* (2003) also reported that when low gravity wort supplementations were carried out with both peptides and amino acids as the sole nitrogen sources, the similar higher biomass, attenuation rate, ethanol production and improved fermentation performance were observed in these fermentations compared to the controls. Also, the viability of the cells, grown in simple peptides and amino acids, was greater than that of cells grown in ammonium ions (Da Cruz *et al.* 2003). This observation is further proof that brewing yeast can also survive and proliferate by metabolizing simple peptides, which provide the same nitrogen efficiency as media rich in amino acids. Additionally, they deduced that when the percentage of dissolved sugars in the medium increased, fermentations supplied with peptides were more efficient than those supplemented with amino acids, in terms of fermentation period, maximum cell number, attenuation rate and alcohol production (Da Cruz *et al.* 2003). The fermentations supplemented with amino acids appeared to decrease biomass synthesis inducing an effect on yeast performance and resulting in the extinction of the secondary growth phase, probably due to the loss of yeast viability (Da Cruz *et al.* 2003). They suggested that the utilization of nitrogen from a complex source is due the combination of the range of permeases present, their specificity and feedback inhibition effects resulting from the composition of the yeast intracellular amino acid pools (Da Cruz *et al.* 2003). These results showed that with an increase in wort gravity, yeast cells prefer more complex nitrogen sources, probably to overcome the stress/gravity related effects and to enhance their fermentation performance. These results also constitute another index that yeast fermentative activity is facilitated by the existence of small peptides in its growth medium.

All the above findings suggest that there is a vital link between assimilation of non-peptide nitrogen sources and the uptake of oligopeptides and no inhibition on the uptake of small peptides takes place when both amino acids and small peptides coexist in the fermentation medium. It can be concluded that as the nitrogen complexity of the yeast growth medium increases, the utilization rate of all nitrogen sources increases in harmony with the result that there are difficulties in determining exactly which nitrogen source was utilized first. Thus, it is suggested that utilization of small peptides from wort may

actually occur at the same time as the uptake of Groups A and B amino acids. Providing the yeast with excess nitrogen nutrient diversity permits cells to expend less energy to synthesize the necessary components for yeast metabolism, hence faster growth rates are achieved. All this information is important for the brewer because it shows the significance of the malting and mashing processes in ensuring adequate quantities of amino acids and small peptides in wort.

4.5 Amino acid wort supplementations

As described in the Results Section **3.3**, supplementation of wort with single amino acids had both beneficial and inhibitory effects on yeast fermentative ability. Based on single nitrogen wort supplementation trials with both two and five times more than the natural concentration of lysine and arginine, these two wort supplements were shown to be advantageous wort nitrogen supplements, since they reduced the fermentation time from 96h to 48h and 67h, respectively. Such reductions in the fermentation period were obviously induced by the stimulatory effect that each of these amino acids had on the yeast growth, resulting in higher levels of yeast cells in suspension compared to the control fermentations. In more detail, the maximum suspended cell number in both supplementations was by $3x10^7$cells/ml higher than those obtained during the control fermentations with the direct result that more yeast cells were present in the fermentation medium utilizing more rapidly the available fermentable sugars. The higher cell number appears to be a beneficial effect of the "good quality" wort FAN enrichment. More specifically, during the first 20-24h of fermentation, maximum cell numbers were observed and simultaneously the FAN utilization rate was at the highest degree, leaving after that point very small amounts of nitrogenous compounds to be used during the rest of the fermentation.

Conversely, the other two single wort nitrogen supplements tested, methionine and ammonium sulphate, caused prolongation of the normal time taken for yeast to reach the desired cessation fermentation attenuation limit from 96h to 103h and 212h, respectively. Furthermore, these two nitrogen supplements provided lower maximum numbers of suspended yeast cells compared to the non-supplemented wort experiments. To be more specific, during the methionine and ammonia wort supplementations, the maximum cell numbers obtained were calculated to be $7.2x10^7$cells/ml and $9.5x10^7$cells/ml, respectively, while during the un-supplemented (control) trials that were carried out at the

same time, the maximum cell number was $10x10^7$cells/ml. It seems that these two single wort nitrogen supplements did not facilitate any amplified yeast proliferation with a direct effect being a slower sugar attenuation rate. In addition, their supplementation in wort appeared to induce a negative effect on yeast multiplication, leaving high levels of unused Group 3 amino acids, such as glycine and alanine. O'Connor (1989), O'Connor and Ingledew (1989) and O'Connor *et al.* (1991) reported that in conditions under which nitrogen is in excess, a larger amount of FAN remains unused. This finding agrees partly with my findings from the supplementation trials conducted with excess of ammonium ions and methionine where, as mentioned above, higher FAN levels remained unused compared to the control fermentations.

Lysine supplementations produced a larger final yeast crop compared to the control fermentations and all the other supplementation trials. This fermentation derivative index was used as a comparative factor in order to identify if this particular single nitrogen wort supplement gave rise to a significant difference regarding the yeast fermentative activity compared to the control and the other supplemented fermentations conducted. Therefore, statistical comparison tests (**Table 27)** were carried out in order to see if the differences resulted in the final levels of produced dry yeast crop were significant or not. Also if the effect derived by supplementation was significant or not. The statistical test used for the purpose was the Tails 2 (two-tailed test), Type 1 (paired data) *t*-test. The *t*-test *P* values that were statistically calculated by the comparison of the lysine single supplementations with the rest of the supplementation tests and control fermentations conducted show that there is always a probability that these sets of results are different and all the estimated probability percentages are less than 5%. It is concluded that lysine wort enrichment is significantly better than all the other single nitrogen compounds supplemented and un-supplemented fermentations. Additionally, it was also shown statistically in the Results Section ***3.4.1*** (**Figures 72** and **73**) that lysine wort enhancement had the most important and significant influence by 95% on the rate of fermentation also pitched with the same lager yeast strain SC3.

Table 27: The *t*-test *P* values derived by comparing the final yeast crop levels produced during lysine supplementations to the control and other supplemented fermentations.

Dry Biomass (g)	x5 Lys	x5 Arg	Dry Biomass (g)	x5 Lys	x5 Meth
Cylinder 1	30.40	25.60	Cylinder 1	30.40	20.04
Cylinder 2	30.31	26.48	Cylinder 2	30.31	20.34
Cylinder 3	30.30	26.55	Cylinder 3	30.30	20.40
t-Test *P*	**0.67%**	**Significant**	*t*-Test *P*	**0.02%**	**Significant**
Dry Biomass (g)	**x5 Lys**	**x2 NH3**	**Dry Biomass (g)**	**x5 Lys**	**Control**
Cylinder 1	30.40	20.29	Cylinder 1	30.40	22.55
Cylinder 2	30.31	23.61	Cylinder 2	30.31	23.93
Cylinder 3	30.30	25.75	Cylinder 3	30.30	23.68
t-Test *P*	**4.82%**	**Significant**	*t*-Test *P*	**0.43%**	**Significant**

Furthermore, lysine supplementations were shown to have given rise to an increased mean ethanol yield compared to the control and other single supplemented fermentations. However, in order to appreciate if this difference was significant or not, another set of *t*-tests was also conducted this time for total ABV% comparison purposes. The results of these statistical analyses are illustrated in the following table (**Table 28**).

Table 28: The *t*-test *P* values derived by comparing the total ABV% levels produced during lysine supplementations to the control and other supplemented fermentations.

Ethanol %	x5 Lys	x5 Arg	Ethanol %	x5 Lys	x5 Meth
Cylinder 1	7.434	6.640	Cylinder 1	7.434	6.747
Cylinder 2	7.556	6.882	Cylinder 2	7.556	6.094
Cylinder 3	7.298	6.909	Cylinder 3	7.298	6.370
t-Test *P*	**3.56%**	**Significant**	*t*-Test *P*	**4.64%**	**Significant**
Ethanol %	**x5 Lys**	**x2 NH3**	**Ethanol %**	**x5 Lys**	**Control**
Cylinder 1	7.434	6.604	Cylinder 1	7.434	6.612
Cylinder 2	7.556	6.766	Cylinder 2	7.556	6.318
Cylinder 3	7.298	6.827	Cylinder 3	7.298	6.231
t-Test *P*	**2.55%**	**Significant**	*t*-Test *P*	**1.31%**	**Significant**

Once again, the *P* values obtained by the statistical analysis of the ABV% levels show that the difference in the ethanol levels produced at the end of the fermentations enriched with excess lysine (x5) compared to the ethanol levels produced at the end of the other

supplemented and control fermentations was significant because all the *P* values were smaller than 5%. Again, it can be concluded that lysine supplementation of the wort type used for my fermentation experiments was beneficial for the rate of fermentation and for other factors directly affecting the quality of the final product.

Thomas and Ingledew (1992) carried out a similar series of nitrogenous material supplementations but the fermentation medium that was enriched with various amino acids was wheat mash, since the amount of FAN present in wheat mash is too low to promote yeast growth and fermentation at the fastest rate. They also concluded that not all amino acids appeared to be equally effective in enhancing yeast multiplication and fermentation rate. Surprisingly, when the unfermented wheat mash was supplemented with exogenous lysine, the fermentation time was prolonged significantly because yeast viability was greatly reduced and moreover the lysine supplement was found to induce an inhibitory effect on yeast proliferation (Thomas and Ingledew, 1992). Moreover, Schultz and Pomper (1948) and later Watson (1976) reported that lysine cannot serve yeast growth as sole source of assimilable nitrogen. Additionally, Bourgeois (1969 and 1976) reported that lysine inhibits the growth of *Saccharomyces cerevisiae.* Hammer *et al.* (1991) stated that *Saccharomyces cerevisiae* can grow with lysine as the sole carbon source, but not as nitrogen source.

However, Thomas and Ingledew (1990) suggested that since wheat mashes are very poor in amino acid sources, then inhibition of yeast growth during lysine supplementation may be related with poor growth rates imposed by the low levels of FAN in wheat mashes. Therefore, these researchers decided to enrich the FAN content of the wheat mashes with various other nitrogen supplements and they surprisingly found that the inhibition caused by lysine was relieved, the fermentation rate was greatly improved and the detrimental low viability effects disappeared. Asparagine, arginine, glutamine, methionine, histidine and ammonia, all these extra nitrogen supplements either reduced or eliminated completely the lysine inhibitory effect (Thomas and Ingledew, 1990). Among all these wort nitrogenous supplements, arginine and methionine facilitated virtually complete uptake of added lysine. The other interesting effect observed was when the wheat mashes were supplemented with only ammonium ions and lysine and the mashes were exhausted of ammonium ions during fermentation, lysine partially induced inhibition in yeast biomass production (Thomas and Ingledew, 1990). When however, the ammonia concentration was above the limiting range, lysine appeared to facilitate the biomass yield (Thomas and Ingledew, 1990).

These findings suggest that the lysine negative effect on yeast growth and viability is induced when the other available nitrogen sources are limiting or close to depletion. Alternatively, when the fermentation medium is rich in free amino nitrogen, then the supplementation of lysine is beneficial through a synergistic effect and it can stimulate both fast fermentation rates and elevated numbers of yeast cells, exactly as occured during my fermentation experiments in which the unfermented wort was supplemented with lysine. Also lysine, during the multiple complex supplementations conducted, was shown to have functioned positively via a synergistic nitrogenous material pathway and to be the most important amino acid in terms of faster fermentation completion and the achievement of target wort gravity. However, Sumrada and Cooper (1976) reported that lysine inhibits the growth of *Saccharomyces cerevisiae* when the medium is nitrogen deficient. The same researchers (Sumrada and Cooper, 1976 and 1978) reported that lysine can be used in the presence of other nitrogenous materials and furthermore Bourgeois (1969) reported that lysine is readily utilized by yeast cells grown in complex fermentation media. Woodward and Cirillo, (1977) observed that lysine can normally be utilized in the absence of other sources of assimilable nitrogen. ter Schure *et al.* (2000) demonstrated that when good nitrogen sources are added in the medium of yeast cells growing on poor nitrogen sources, the transcription of some genes involved in the utilization of the poorer nitrogen source is repressed and their corresponding products are inactivated or degraded. This physiological response of gene expression inactivation in reaction to the nitrogen source present in the medium is called nitrogen catabolite repression (ter Schure *et al.* 2000).

The transcription repression of permease encoding genes and the selective inactivation and subsequent degradation of existing permeases upon addition of good nitrogen sources in the growth medium prevents uptake of the poorer ones (e.g. proline) and it usually takes some time for this process to be complete (ter Schure *et al.* 2000). These two mechanisms ensure that the best nitrogen source for growth is selected (ter Schure *et al.* 2000). Marder *et al.* (1977) carried out a supplementation experiment with various mixtures of lysine and leucine and the *Saccharomyces cerevisiae* strain used for the pitching of the fermentations was a *lys, leu* auxotroph. Based on their findings they observed that the minimal concentration of lysine needed for triggering maximum yeast growth was only 15mg/L, whilst for the same maximum concentration to be reached with leucine supplementation was double the amount of the lysine required (Marder *et al.* 1977). This observation indicates that even very low concentrations of lysine can trigger

elevated biomass levels in the presence of other available nitrogen sources and obviously improved fermentation performance. All these statements, supported by the data discussed above, are in agreement with my lysine supplementation results, since both supplementation experiments of the unfermented wort with two times and five times of lysine's natural wort concentration had the effect of decreasing the fermentation period from 96 to 48h. Additionally, the Minitab statistical analysis showed that lysine was found to be the only amino acid that had a significant beneficial effect on the rate of sugar attenuation confirming my hypothesis that lysine is a key amino acid for facilitating the brewer's yeast's metabolic activities. Thomas and Ingledew (1992), after completing their wheat mash supplementations, reported that lysine inhibition was not clear if it was caused by α–aminoadipic semialaldehyde or by its oxidation intermediate product, α–aminoadipic acid. However, α–aminoadipic acid has been shown to inhibit yeast growth (Bourgeois, 1976) but this inhibitory effect can be alleviated by simultaneous addition of arginine, methionine and aspartic acid (Winston and Bhattacharjee, 1982). Thus, it can be hypothesized that a part of the alleviating process of lysine-induced inhibition may involve the interaction of the other added nitrogen sources with the possible toxic produced derivatives of lysine metabolism.

Furthermore, Thomas and Ingledew (1992) observed that elevated levels of arginine in the wheat mash medium were beneficial for yeast performance during fermentation. In more detail, from all the nitrogen supplements tested, arginine was found to be the most effective amino acid in promoting the fermentation rate of the wheat mash by triggering rapid cell multiplication and also aiding retention of high viability of yeast cells until the end of fermentation (Thomas and Ingledew, 1992). In addition, as discussed above, arginine was found to be the most ideal amino acid for alleviating the inhibition caused by the single addition of lysine in the wheat medium (Thomas and Ingledew, 1992). During my wort supplementation experiments, arginine as a single wort nitrogen supplement, was also found to trigger a faster fermentation rate than that observed by the control fermentations. It was also shown that arginine supplementation with five times its initial natural wort concentration triggered higher maximum cell growth, higher levels of both total yeast crop and ethanol produced at the end of the fermentation trials compared to the control fermentations. The same effects were observed when the supplementations were carried out with two times the initial natural wort concentration of arginine, exactly as occurred with the lysine and methionine supplements.

Similarly, Paik *et al.* (1991) observed that glutamic acid supplementation of a malt extract wort had a stimulatory effect on yeast metabolic activity. However, they realized that further wort supplementations at various higher concentrations with the same medium supplement did not have any significant effect on yeast performance. They suggested that the nitrogen requirements during fermentation can be saturated, because after adding 200 and 300mg/L glutamic acid to the medium, the fermentation time for completion was only reduced by 2h (Paik *et al.* 1991). Enari (1974) and Enebo and Johnsson (1965) also reported that by increasing the initial FAN levels of wort above a certain value has little or no beneficial effect on the fermentation profile. These results correlate with my single supplemented fermentation data, where worts supplemented with two and five times the original wort concentration of lysine, arginine and methionine supported more or less the same fermentation performance regardless of the final effect on attenuation rate. This means that FAN levels may reach a saturation point above which there are no further positive effects on overall yeast performance.

Marder *et al.* (1977) showed that *Saccharomyces cerevisiae* growing in an artificial fermentation medium consisting of Lys-Gly dipeptides as the sole nitrogen source exhibited a prolonged lag phase in the addition of the tripeptide $(Meth)_3$ (tripeptide consisting of three molecules of the amino acid methionine). They also reported that the additional peptides with two and three methionine amino acid residues induced a strong inhibition of the uptake of Lys-Gly. Thus, they concluded that these yeast growth delays and the observed inhibition of uptake between these two peptides arose from an interaction competition at the nitrogen source transport level (Marder *et al.* 1977). These observations may be correlated with the effect that the methionine supplementations had on the utilization of glycine during my methionine supplemented fermentations, where significant amounts of this Group C amino acid remained unconsumed in the fermented wort and also the fermentation period was prolonged in methionine excess. Furthermore, based on the theory of ter Schure *et al.* (2000) about the nitrogen catabolite repression, it can be assumed that the addition of excess methionine to wort may be the main reason for incomplete utilization of glycine via inactivation of the glycine permease indicating that glycine is a poor nitrogen source for yeast growth and metabolism. However, during the complex amino acid and ammonia wort supplementation combination trials, methionine was shown to have reinforced the yeast fermentative activity synergistically with the other nitrogen supplements by reducing the period of the fermentation process. Moreover, as previously discussed, Thomas and Ingledew (1992) during their

supplementation experiments observed that methionine addition in the lysine supplemented wheat mash facilitated yeast growth and stimulated the uptake of the additional lysine.

Two years previously the same authors, Thomas and Ingledew (1990), observed that addition of exogenous protease to the wheat mash resulted in a decrease in fermentation time from 120 to 72h. The stimulatory effect of the protease resulted in an increase in FAN content to support greater yeast growth, via the hydrolysis of proteins into simple peptides and amino acids (Thomas and Ingledew, 1990). Supplementation of the wheat mashes with mixtures of casamino acids and yeast extracts resulted in faster fermentation rates than those observed during control fermentations (Thomas and Ingledew, 1990). Thorne (1949) examined the growth rates of *Saccharomyces cerevisiae* on media supplemented with simple ammonium ions, single amino acid nitrogen sources and mixed amino acids. From this series of experiments, it was found that media containing mixtures of amino acids were superior compared to the fermentation media supplemented with simple ammonia as a sole source of assimilable nitrogen (Thorne, 1949). Thorne (1949) suggested that obviously there is an ideal wort amino acid and ammonium ions combination to facilitate the fermentative ability of yeast so that high quality beer is produced in the fastest possible time. Such an assimilable nitrogen composition was statistically analyzed and established for the lager yeast strain SC3, shown in **Figures 87a** and **87b**. However, Jones and Ingledew (1994) reported that the fermentation time with ammonium ions used as nitrogen supplements twice the initial concentration, decreased from 9 days to 4 days. On the other hand, during my ammonia supplementation experiments the fermentation time was prolonged from 96 to 212h, meaning that the single ammonium ion addition had a deleterious effect on lager yeast strain SC3, metabolic and fermentative activity. In addition, Jones and Ingledew (1994) observed that ammonia supplementation led to the excretion of glycine in the fermentation medium, where during my ammonia supplemented fermentations, complete absorption of both alanine and glycine was inhibited and high levels of these two wort nitrogenous components remained unexploited at the end of the fermentations.

Such an effect was probably induced again due to nitrogen catabolite repression, where yeast used more readily the nitrogen sources to cover its metabolic requirements and inhibited the permease system for these two poor Group C nitrogen wort sources. Moreover, the yeast crop, the ethanol and VDK levels produced at the end of the fermentation supplemented with ammonia were inferior even to those produced at end of

the un-supplemented fermentations. In addition, ter Schure *et al.* (2000) reported that the ammonia transporters also function as the retainers of endogenous ammonium ions during growth of the yeast on sources other than ammonia. At very high ammonia concentrations, the *MEP* gene expression is repressed due to feedback inhibition and the ammonium ion transporters are blocked and cease functioning (ter Schure *et al.* 2000). The *MEP* gene encodes a specific, high-capacity NH_4^+ transporter protein known as Mep1p in *Saccharomyces cerevisiae* (ter Schure *et al.* 2000). This finding probably explains the yeast fermentative behaviour under conditions of excess ammonia, where the fermentation time during the supplemented trials was prolonged from 96 to 212h, compared to the control fermentations.

Diacetyl production is strongly affected by the levels of FAN in wort (Pugh *et al.* 1997). High levels of diacetyl are produced in beer during fermentations with high initial wort FAN content (Pugh *et al.* 1997). This can be explained by the order of amino acid uptake by yeast according to their classification (Pugh *et al.* 1997). For instance, Pugh *et al.* (1997) reported that during the uptake of Group A amino acids, yeast synthesizes valine. Since, α–acetolactate, the precursor of diacetyl, is a by-product of valine synthesis; diacetyl is produced with simultaneous formation of valine (Pugh *et al.* 1997) **(Figure 101)**.

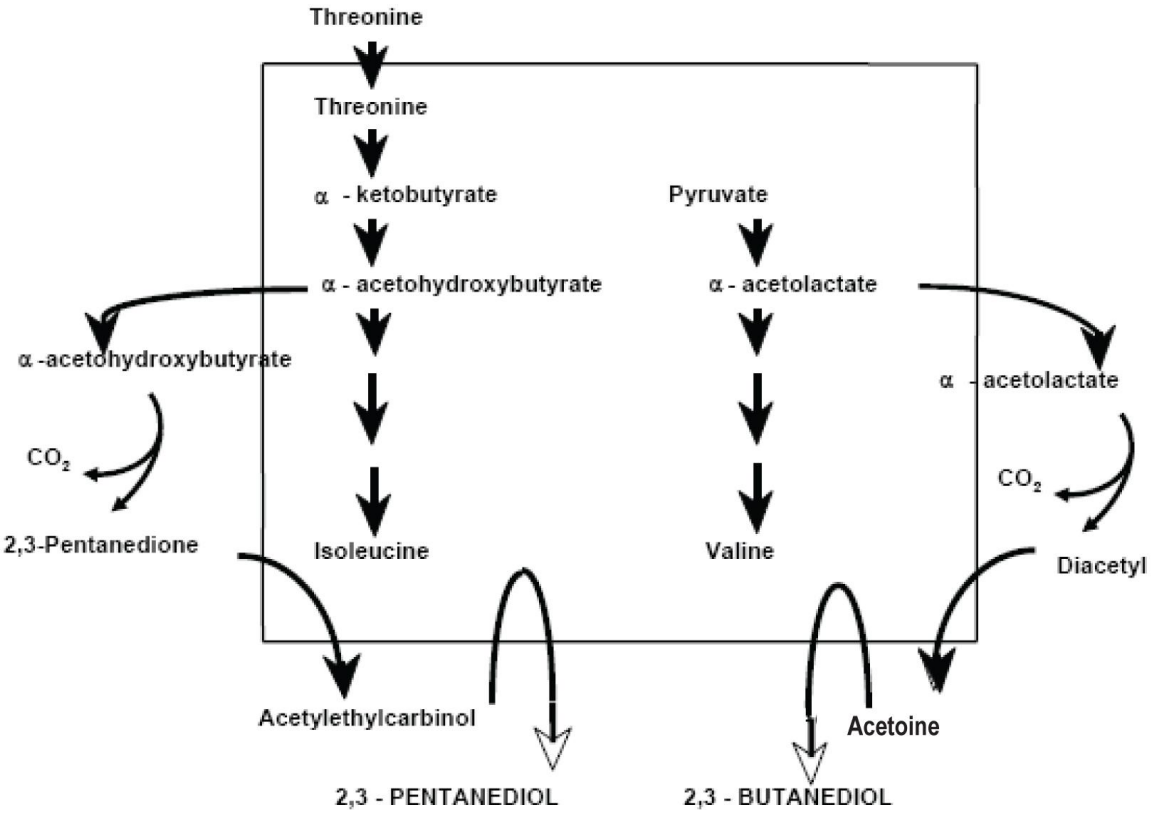


Figure 101: The two metabolic pathways for diacetyl and 2,3 pentanedione formation (Source: www.brewingtechniques.com)

For that reason, high FAN worts that contain high Group A amino acid levels, result in fermentations with a longer period of valine synthesis and hence more diacetyl production (Pugh *et al.* 1997). It was also found that supplementation of wort with Group A amino acids serine and asparagine, increases the diacetyl production by delaying the uptake of valine from wort and extending the period of valine synthesis (Pugh *et al.* 1997). In all likelihood, the same effect also occurred during my single supplementations conducted with lysine and arginine (both belong in Group A), where elevated levels of total diacetyl were detected in the samples of fully fermented wort. In more detail, the elevated 2,3 butanedione levels found in the final fermented wort were formed most likely due to the delayed uptake of valine induced by the addition of lysine and arginine with the consequential result being the extension of valine and obviously α–acetolactate synthesis. The same observation for lysine supplementation was also confirmed by examining the statistical analysis of supplementation results (**Figure 81**), where it was proved that by increasing the lysine concentration twice more than its natural wort levels, the final beer diacetyl levels increased. The same effect was also noted when histidine was used as a

wort nitrogen supplement (**Figure 81**). On the other hand, when wort was supplemented with valine and leucine, diacetyl levels were reduced. Based on these observations, it can be assumed that when the fermentation medium was supplemented with amino acid combinations that contained valine and/or leucine twice as their original wort concentration, then the yeast synthesis of these two amino acids was suppressed, probably by a feedback inhibition mechanism since both of these nitrogenous wort supplements were present in excess in the medium. Such a suppression of valine and leucine production also had a consequential effect on the suppression of diacetyl precursor formation, α–acetolactate, as shown in **Figure 102**. Moreover, Nakatani *et al.* (1984) confirm this observation by reporting that when valine is assimilated by yeast, the formation of total diacetyl is strongly suppressed, meaning that no more valine is needed to be synthesized since the medium is saturated with this particular amino acid. In addition, acetohydroxyacid synthase (AHAS), the enzyme that catalyzes the first common step in the synthesis of branched-chain amino acids, valine, leucine and isoleucine (**Figure 102**) is repressed by allosteric feedback inhibition of valine (Tittmann *et al.* 2005).

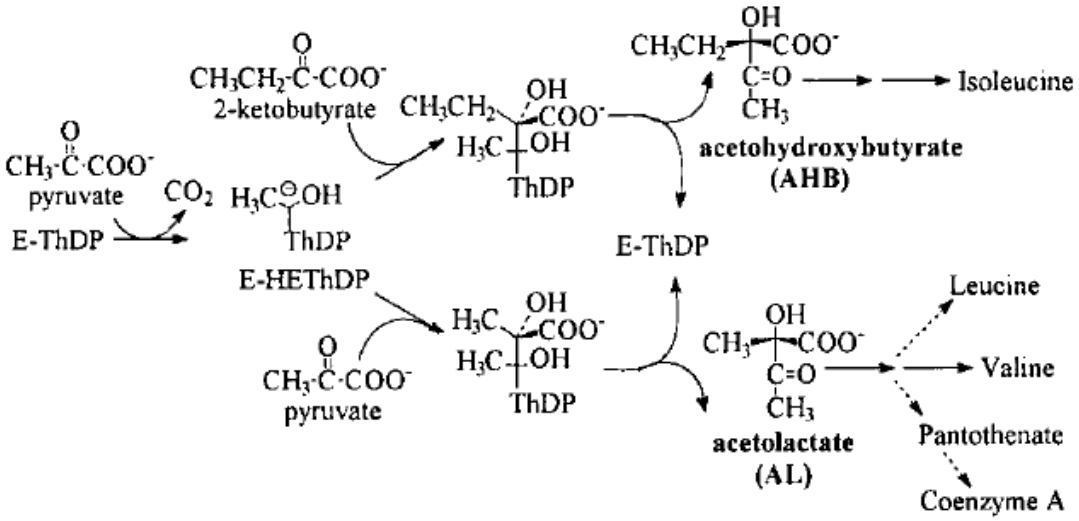


Figure 102: The metabolic reactions catalyzed by acetohydroxyacid synthase (AHAS) (Source: www.biochemj.org)

In addition, as shown in **Figure 85**, isoleucine and valine were found to be the only two amino acids that significantly affect 2,3 pentanedione production. In other words, by doubling the natural wort concentration of these two amino acids, the levels of 2,3

pentanedione decrease. Thus, it can be assumed that since the medium is saturated in isoleucine no further synthesis of this nitrogenous nutrient is needed and obviously, no acetohydroxybutyrate is formed (and then to be decarboxylated to produce 2,3 pentanedione). In addition, AHAS, as mentioned above, is repressed by allosteric feedback inhibition of valine (Tittmann *et al.* 2005). If valine is present in the medium, repression of AHAS leads to depletion of isoleucine resulting in the simultaneous exhaustion of acetohydroxybutyrate and obviously 2,3 pentanedione. Nakatani *et al.* (1984) also reported that the assimilation of isoleucine suppresses the total 2,3 pentanedione and that valine showed a significant suppressive effect on 2,3 pentanedione formation. As described above, AHAS catalyzes the first common step in the synthesis of the amino acids valine, leucine and isoleucine (**Figure 102**) (Tittmann *et al.* 2005). This enzyme belongs to a homologous family of thiamin diphosphate (ThDP)-dependent enzymes, whose initial step is the decarboxylation of pyruvate (Tittmann *et al.* 2005). In the process catalyzed by AHAS, the decarboxylation of pyruvate to form the hydroxyethyl-ThDP anion/enamine (HEThDP) is followed by the specific condensation of the intermediate with a second aliphatic ketoacid to form an acetohydroxyacid (Tittmann *et al.* 2005). The competition between the two alternative second substrates, 2-ketobutyrate and pyruvate, leads to partition of the flux through AHAS between acetohydroxybutyrate and acetolactate (Tittmann *et al.* 2005). So, the relative rates of synthesis of isoleucine and of valine and leucine, are determined respectively (Tittmann *et al.* 2005). With this in mind, it is believed that the increase detected in the second vicinal diketone, 2,3 pentanedione, after wort supplementation with lysine and or arginine, may be due to the parallel increased flux through the isoleucine synthesis pathway induced by the stimulatory action that these two nitrogen wort supplements had on the enzyme AHAS. In more detail, it can be assumed that lysine and arginine wort enrichment facilitated the enzymatic activity of AHAS in such a way that both metabolic routes were simultaneously activated so that more α–acetolactate and acetohydroxybutyrate were produced and consequentially more diacetyl and 2,3 pentanedione.

Another possible explanation for the increased formation of vicinal diketones at the end of the lysine and arginine supplementation experiments could be the increase of the yeast biomass. Additionally, when fermentation attenuation was complete fermentation vessels were immediately cooled to 4^{o}C, allowing the yeast cells to settle. Thus, it is believed

that if the fermentation experiments were permitted to proceed for a further 24h, the VDK levels would have been lower by being re-absorbed into the yeast cells. Wainwright (1973) reported that warm conditioning of beer (12-16^{o}C) after fermentation allows yeast to reabsorb diacetyl from the medium. However, if yeast is separated from the final fermented wort (by use of a very flocculent yeast culture or use of a centrifuge) before the complete decomposition of α–acetolactate, diacetyl is produced spontaneously and no yeast cells are present to absorb it and consequentially it accumulates in the beer (Barton and Slaughter, 1992).

Ammonia and methionine supplementations, on the other hand, did not trigger any elevation in the final concentrations of the vicinal diketones and their precursors. Similarly, Barton and Slaughter (1992) also found that ammonium ion supplements induced a 90% decrease in the final VDK levels, compared to their control samples. A possible explanation for such an effect is that ammonium ions in excess inhibit the AHAS activity and thus lower levels of VDKs were produced (Barton and Slaughter, 1992). Both ammonium ions and methionine may be blocking the AHAS active site and thus deactivating the catalytic functionality of the enzyme (Barton and Slaughter, 1992). Finally, neither methionine nor ammonia supplements stimulated the synthesis of elevated final VDK levels probably because none of them induced any stimulatory effect on yeast proliferation. This resulted in very small quantities of α–acetolactate leaking out of the yeast cells into the fermenting wort, which were then slowly, non-enzymatically, decarboxylated into diacetyl (Barton and Slaughter, 1992).

4.6 Mashing trials

As described in the Materials and Methods Sections ***2.6.1*** and ***2.6.2***, two series of mashing experiments were conducted using the same barley malt varieties. In more detail, mashing was first performed at 65^{o}C, in order to determine the FAN content of the mashes. The same malts were then mashed at 4^{o}C, in order to investigate the actual percentage of FAN produced during malting by the breakdown of the barley endosperm proteins and the percentage formed during mashing after proteolysis of the mash proteins by the mash exopeptidases. Consequently, by repeating the same mashing process but this time at 4^{o}C, the mash proteases remained inactive at this temperature and with almost no mashing proteolytic activity. After the completion of these trials, it was observed that

88% of the mean FAN content analyzed already existed in the malted grain. In other words, the FAN content produced during malting constitutes 88% of the total FAN concentration found in wort and only 12% is produced during mashing. Barrett and Kirsop (1971) carried out a similar mashing experiment, where the level of malt soluble nitrogen were measured at 4^{o}C in order to inactivate the proteolytic endosperm enzymes and then extraction of the enzyme-free product took place. The quantity of FAN solubilized during mashing was measured by comparing the amount of FAN present in the malt with that present in the wort, after mashing at 65^{o}C. In addition, in order for satisfactory enzyme inactivation to be achieved, malt grist was treated with boiling aqueous ethanol (80% v/v) (Barrett and Kirsop, 1971). Their results showed that on average half of wort FAN was derived during barley germination and the other half during mashing (Barrett and Kirsop, 1971). These observations represent a large discrepancy to my findings since, as it was previously discussed, only 12% of wort FAN is synthesized during mashing at 65^{o}C. These data differences lead us to believe that the boiling ethanol solution that was used for enzyme inactivation was not as effective as the 4^{o}C mashing used for my experiments. In addition, Lie (1973) reported that the greater part of FAN (70%) found in wort is pre-formed in malt at low temperatures, whilst the remaining part of nitrogenous materials (30%), originates from the action of proteases during mashing. The same observations were also made by Taylor and Boyd (1986), who reported that under normal mashing conditions, approximately 30% of FAN in wort derives from mashing proteolysis and the rest already exists in the germinated grain. However, Palmer and Bathgate (1976) suggested that FAN formation during high temperature mashing is minimal. These researchers seem to have experienced the same observations as those in my trials, where the FAN produced during mashing at 65^{o}C varied between 5 to 20% of the total produced wort FAN. Furthermore, the same authors also found that the FAN levels in wort are correlated with those found in malt. They also reported that the use of a high FAN malt resulted in a high FAN wort content and vice versa. This observation was also confirmed in my experiments, where high nitrogen content malt varieties resulted in high FAN content worts. Additionally, such an observation also confirms my suggestion that the FAN levels produced during the mashing step at high temperature are negligible and the majority of nitrogenous materials that constitute yeast assimilable nitrogen are formed in the barley germination stage of malting. Ratnavathi *et al.* (2000) also discovered during their experiments, conducted with sorghum mashes, that the removal of the grain husks of malted sorghum resulted in a

very significant reduction of wort FAN, sometimes FAN was reduced to 10.2mg/g, depending on the sorghum variety. This is another confirmation indicating that the highest unfermented wort FAN content is a derivative of the malting process.

Despite the fact that Taylor and Boyd (1986) and others have reported that HWE and FAN are two important parameters for predicting the quality of beer, my results showed that there is no correlation between the FAN and the HWE malt content. In other words, a malt cultivar with high HWE value does not necessary exhibit a high FAN content and that the resulting beer will have good quality and stability attributes. Taylor and Boyd (1986) also note that during sorghum mashing, the increase in the low molecular weight nitrogen (an alternative term for FAN) content was relatively small and even after only 2h mashing, this fraction still constituted only 10.6 to 12.4% of the total FAN content. Enari (1974) also reports that, based on European brewing data, the FAN content in unfermented wort constitutes 12% of the total alpha nitrogen content in the malted barley. These results are in agreement with mine, where it was found that only 12% of the total FAN content found in the unfermented wort is produced during mashing. Additionally, it was found, by analyzing the spectrum of amino acids in the malts and then in mashes of these malts, 63% of nitrogen derived from Group A amino acids is formed during barley germination. As for the rest of the assimilable nitrogen that derives from Groups B and C, only 26.5% is produced during mashing by the activation of mash proteolyitc enzymes. The same observation is also valid for the only component of Group D, proline. Similarly, when the ammonia wort content was analysed before and after mashing, it was found that 75% of ammonium ions were already formed in the malted barley prior to mashing at 65^{o}C. Mandl *et al.* (1972) reported that the ammonia portion of the wort nitrogen, which is formed by decomposition of amides, is highest at the beginning of malting (3rd to 5th day) and thereafter its concentration decreases gradually and reduces during a prolonged germination time.

In general, the total malt FAN increases considerably during the first days of germination and reaches a maximum level between the 7th and 8th day of germination (Mandl *et al*. 1972). Maximal peptidase activity is normally not obtained within the customary germination time (Mandl *et al*. 1972). The dipeptidase shows the highest activity during the 4th or 5th day (Mandl *et al*. 1972). While the enzymatic cleavage of a tripeptide gradually increases from the first day onwards, the cleavage of a dipeptide commences only during the 3rd day of malting (Mandl *et al*. 1972). It has also been reported that high levels of protein in barley are responsible for the development of greater proteolytic

activity during barley germination (Mandl *et al*. 1972). The proteins are degraded to amino acids and small peptides and they are transported to the acrospire (Mandl *et al*. 1972). This migration reaches a maximum between the 2nd and 6th day and particularly during the 4th and 5th day of germination (Mandl *et al*. 1972). In addition to enzymatic proteolysis, synthesis and re-arrangement of amino acids are also taking place (Mandl *et al*. 1972). Based on my results, 50% of the amino acids aspartate, glutamate, methionine and lysine, found in the mashes, were synthesized during germination and the other half during mashing. Aspartic acid malt content increases continuously and its highest value is obtained between the 8th and 10th day of malting, while lysine concentration increases considerably during the first days of germination and reaches a maximum concentration between the 8th and 9th day (Mandl *et al*. 1972). In addition, glutamic acid shows highest value during the 2nd and 3rd day, whilst highest values of methionine are obtained on the 3rd day of germination (Mandl *et al*. 1972).

According to my data in **Figure 99**, 67.5% of the amino acids serine, arginine, leucine and isoleucine were already formed in the malted grain during germination, where the remainder was produced when the grist samples were mashed. Arginine increases considerably during the first day of germination and reached a maximum between the 7th and 8th day and that concentration commences to decrease after the 8th day, while the increase in serine is particularly high during the first two days of malting (Mandl *et al*. 1972). However, highest values are obtained between the 7th and 9th day of barley germination (Mandl *et al*. 1972). Maximum concentration of leucine is achieved between the 7th and 10th day of germination. Approximately 73% of the amino acids asparagine, glutamine, threonine, glycine, proline, phenylalanine, histidine, tyrosine and isoleucine were found to be present in the malted barley. Histidine increases steadily up to the 10th day of germination while its portion of the wort nitrogen reaches a maximum during the 8th day of germination (Mandl *et al*. 1972). Threonine increases rapidly during the first days of germination, however, its rate of increase slows down later. Highest concentration is obtained between the 7th and 9th day (Mandl *et al*. 1972). Glycine increases continuously up to the 8th day of germination (Mandl *et al*. 1972). Isoleucine shows considerable increase during the first three days. Highest concentration is achieved between the 7th and 8th day (Mandl *et al.* 1972). Tyrosine, initially shows a considerable increase and highest concentration is reached on the 8th and 9th day (Mandl *et al.* 1972). Phenylalanine content of the malt reaches maximum values on the 7th or 8th day of germination (Mandl *et al.* 1972). Finally, the proline concentration in the malt

increases continuously up to the 10^{th} day and only this amino acid fails to reach a maximum level even after ten days of germination so that its level can be considered as a criterion of the germination time (Mandl *et al.* 1972).

The three remaining amino acids alanine, valine and tryptophan were found that only 20% of their concentration is a product of the mashing process, while 80% was preformed during grain malting. According to the findings of Mandl *et al*. (1972), the tryptophan content of the malt increases continuously during germination and the highest values are obtained between the 7^{th} and 10^{th} day. In addition, alanine increases very rapidly during the first days of germination and reaches a maximum concentration between the 8^{th} and 10^{th} day (Mandl *et al.* 1972). Valine increases considerably during the first days of germination. Highest concentration is obtained between the 8^{th} and 10^{th} day (Mandl *et al.* 1972).

Unmalted barley contains only traces of amino acids (Mandl *et al*. 1972). There is however, a considerable increase during malting until a maximum concentration is reached followed by a decrease (Mandl *et al.* 1972). This reduction is most probably due to the acrospire requirements for larger amounts of nitrogenous compounds than being produced during protein degradation (Mandl *et al.* 1972). Furthermore, the rootlets and shoots retain large quantities of various amino acids (Mandl *et al.* 1972). It should also be mentioned that under-modified and over-modified malts contain less amino acids than normal modified malt (Mandl *et al.* 1972). When using these malt types, it is possible that difficulties will be experienced with the fermentation rates, yeast growth and fusel oil production (Mandl *et al.* 1972). A germination time of at least four days is required in order for malts rich in assimilable nitrogen to be obtained. However, the barley variety is also of considerable importance (Mandl *et al.* 1972). Thus, the degree of modification is probably one of the major attributes indicating the quality of malt before being used for the production of wort that might give rise to sluggish and incomplete fermentation.

In general, the brewer by knowing the amino acid content of a malt type before mashing and commencing fermentation, it is quite possible to predict the fermentation time and also the quality and stability of the beer. Such an effect can be easily achieved by firstly analyzing and establishing the optimal amino acid spectrum that favours the fermentative ability of a particular yeast strain that is always used for the completion of fermentation so that high quality beer devoid of any "off flavours" is produced in the fastest possible time. Hence, by examining the amino acid spectrum of the malt prior to mashing and fermentation, it is a very useful "instrument" for the brewer in order to obtain a good idea

about the fermentation performance and what volatiles are most likely to be synthesized. In addition, the brewer can save funds and valuable time before purchasing a large malt batch to commence brewing. If a small sample of the malt variety that is intended to be bought, is first analyzed for its amino acid-ammonium ion content and it is discovered that will give raise to a great majority of problems regarding the fermentation performance and the quality of the beer produced, this particular malt batch can be rejected by the brewer.

Chapter 5: Conclusion

5.1 Shake flask fermentations

During stirred normal (12^{o}Plato all malt) and high gravity (20^{o}Plato all malt and 20^{o}Plato all malt + 30% glucose) wort fermentations, the ale yeast No 70 assimilated completely only the amino acids lysine, arginine and methionine in order to achieve the appropriate metabolism of the wort fermentable sugars. The rest of the available free amino acids did not undergo significant yeast uptake and high levels remained unmetabolised in the fermented wort. Surprisingly, ammonium ions during the series of high gravity wort fermentations (20^{o}Plato all malt and 20^{o}Plato all malt + 30% glucose) exhibited a greater degree of absorption than Group B and C amino acids, with just ammonia traces detected at the end of these experiments. Particularly, the whole spectrum of Group C amino acids noted almost the same utilization rate as proline. However, during the normal wort gravity (12^{o}Plato all malt) fermentation series, ammonia accumulated in the fermentation medium after 72h fermentation until the completion of the experiment. Similarly, glutamate levels, after the first 24h fermentation, began to accumulate in the medium, regardless of the wort type. In addition, as it was observed by examining sugar catabolism during all the shake flask fermentations conducted, only maltotriose was left unfermented. Hence, it could be concluded that the nitrogen sources, lysine, methione, arginine and ammonia can fully support the metabolic needs of the brewing yeast strain in order for metabolism of the wort saccharides to be sufficiently carried out. Nevertheless, a new perspective was revealed, when the wort oligopeptide content performance was analyzed, during the normal gravity stirred fermentations. The ale yeast strain (No 70) was found to have preferred large molecular weight nitrogen compounds in order to form more utilizable nitrogen wort constituents, rather than using the easily accessible wort's single amino acids. Such yeast behaviour may have derived from the fact that continuous yeast agitation may induce an inhibitory stress effect on its metabolic performance with consequential excretion/secretion of proteolytic enzymes and the resultant nitrogen assimilable products of protein and polypeptide degradation. It is believed that this phenomenon would have been more substantial during high gravity stirred fermentations, since it is well documented that yeast under high gravity conditions is strongly stressed due to osmotic pressure and high ethanol concentration stress factors (Zaragoza and Gancedo, 2000; Stewart, 2001; Hammond *et al.*

2001; Pratt-Marshall *et al.* 2002; Pratt *et al.* 2003) and also the agitation that facilitates this stress effect (Stoupis *et al*. 2002 and 2003). In other words, it is believed that, if the spectrum of wort small peptides would have been also studied, the activity of the extracellular yeast proteases would have been more distinct and emphasized due to the synergism of all the stress factors described above. Thus, it is anticipated that more large peptides and proteins would have been broken down in order for yeast to form more utilizable sources of nitrogen. Once again, it was confirmed that agitation is considered a very significant factor inducing stress to yeast cells during fermentation. Nevertheless, the alternative theory of yeast autolysis and consequential release of intracellular yeast proteases in wort should not be excluded as a possible explanation for the degradation of wort proteins and large molecular weight peptides into more utilizable nitrogenous compounds but would appear to be unlikely. The total nitrogen mass balance calculations that were carried out for the 12^{o}Plato all malt wort, inoculated with the ale strain No 70, shows that 73% of the yeast assimilable nitrogen constitutes small peptides, 14% ammonium ions and the remaining 13%, single amino acids. However when, the nitrogen distribution of the high gravity all malt and adjunct worts was analyzed, it was found that 17% of utilizable nitrogen derives from amino acids, 28.5% from ammonium ions and 54.5% from oligopeptides. Therefore, it was shown that higher gravity worts do not necessarily contain higher levels of amino acids, since ammonia levels detected in 20^{o}Plato worts, were double that found in the normal gravity wort.

5.2 Static fermentations

Unlike the poor and incomplete yeast amino acid utilization performance observed during the stirred fermentations, sufficient amino acid uptake was observed for all the static anaerobic fermentations conducted with the two industrial lager and the two ale yeast strains. The only expected exceptions to this satisfactory assimilation pattern were the wort amino acid, proline and ammonia levels. Exactly the same nitrogen assimilation effects were repeated, when repitched fermentations followed. From my results obtained from the sum of amino acid and ammonia utilization rates observed during static fermentations, the main uncertainty in amino acid groups is to assign an amino acid assimilation order within each group. In other words, while some amino acids may have relatively fixed absorption positions, substantial variations were observed with a number of amino acids when comparisons were made from one fermentation to another. This is likely to be the result of variations in physiological conditions of yeast cells from one fermentation batch to another

and also variations in other fermentation parameters and conditions. On the other hand, Jones (1987) proposed that the uptake of individual wort amino acids during fermentation by yeast occurs in a set sequence, which is almost independent of the fermentations conditions employed. Nevertheless, it was possible, based on my results, to establish a general order of amino acid uptake for both lager and ale yeast strains, which can be seen in **Table 26**. According to my mass balance calculations, the nitrogen distribution of the high gravity adjunct wort type used (15^{o}Plato + 30% VHM syrup), for the completion of the series of static fermentations was almost identical to that observed for the normal gravity all malt wort (12^{o}Plato) used for the shake flask trials.

In addition, it was verified during the static fermentations examined that even in the presence of considerably higher amounts of wort amino acids, the excretion of yeast proteolytic enzymes may not be suppressed. In other words, even in a sufficient FAN content medium, the activity of yeast proteases that break down larger peptides into smaller peptides is not inhibited and yeast cells, apart from the available nitrogen wort sources, are able to also assimilate newly synthesized small peptides. However, the presence of yeast proteolytic enzymes, responsible for protein and polypeptide hydrolysis in a wort with high FAN content, could be the result of cell lysis and consequential enzyme release into the fermentation medium. As discussed in Section **4.4**, a large number of publications (Dalme and Thorne, 1949; Macwilliam and Clapperton, 1969; Clapperton, 1971a; Clapperton, 1971b; Marder *et al.* 1977; Nisbet and Payne, 1979; Ingledew and Patterson, 1999; Patterson and Ingledew, 1999) reported that yeast cells can utilize small peptides in order to accommodate their nitrogen metabolic needs. However, there was a question as to how and when this fraction of the wort utilizable nitrogen content is assimilated. It is believed that this question was fully considered, since it was shown in my results that small peptides and amino acids are simultaneously taken up by the yeast cells, even during the early stages of fermentation when the wort is still rich in single amino acids and ammonium ions. Furthermore, it was also shown that it could be possible for viable, healthy yeast cells, just hours after their inoculation into wort, to commence continuously producing additional assimilable nitrogen components by excreting/secreting proteases that break down the peptide bonds that hold together the building blocks of proteins and other larger peptides. Nevertheless, as already discussed in this document another possibility for such an effect, could be the lysis of yeast cells and the consequential release of the internal yeast proteolytic enzymes into the fermentation environment. The theory that yeast commences to "search" for alternative nitrogen sources and consequently excretes exopeptidases only after all the available wort

FAN reservoirs are exhausted, has been clarified. However, it should be also added that these exopeptidases, inducing the proteolysis of large wort proteins and peptides may have been liberated into wort after the possible lysis of a number of yeast cells during fermentation. Additionally, it can be suggested that the nitrogen limitation is not necessarily a limiting factor in yeast brewing fermentations because it was shown that under these circumstances, fermentation does not cease since yeast continues to excrete/secrete or release following cell lysis, proteolytic enzymes having as a result, large wort molecular weight nitrogen molecules to be converted into smaller assimilable peptides.

5.3 Wort supplementations

According to the results obtained during and after the completion of the series of single supplementations in comparison to control fermentations, lysine and arginine were found to have beneficial effects on the fermentation rate, yeast fermentative performance and proliferation. In other words, increasing the concentration of lysine and arginine in the fermentation medium, prior to yeast pitching, facilitated a healthy and faster fermentation rate compared to un-supplemented fermentations. However, it was also observed that increasing the concentration of these two wort supplements above a certain concentration had little or no positive effect on the fermentation profile. In more detail, by supplementing the wort with two and five times the initial concentration of lysine and arginine, exactly the same effects were achieved. This means that individual wort amino acid levels may reach a saturation point above which there is no further positive effect on yeast performance and metabolism.

My preliminary theory, concerning the effect that lysine wort enrichment had on the rate of fermentation and yeast fermentative ability, was shown to be valid since it was also statistically confirmed that this amino acid was the only wort nitrogen supplement that induced a significant positive effect (more than 95% certain probability) on yeast fermentation performance. Also its wort concentration increase shortened the time needed to achieve complete sugar attenuation. Thus, lysine can be considered to be the main "key amino acid" for stimulating a faster fermentation rate with consequential effects on beer quality and stability. It can be concluded, that the nature of the amino acid used as the wort supplement directly influences the rate of fermentation and also yeast proliferation. Alternatively, when both methionine and ammonium ions were used as single wort nitrogen supplements, lower suspended yeast cell numbers, reduced final yeast crops, lower ethanol

levels and prolonged fermentation times were observed, compared to the control fermentations.

Regarding complex nitrogen supplementation effects, the only amino acids that were statistically analyzed and found that their increase reduced the fermentation rate and also inhibited the fermentation performance of yeast were glutamine and alanine. The increase in the concentration of the Group A amino acid serine, the increase in Group B amino acids valine and histidine and Group D, proline did not have any effect on fermentation rate or yeast fermentation performance. In addition, it was observed that the amino acids valine, leucine, histidine and lysine have a significant effect on the production of diacetyl. In more detail, increase in the concentration of the amino acids histidine and lysine in wort led to an increase in beer diacetyl levels. On the other hand, increases in the wort levels of valine and leucine led to the reduction of the diacetyl level found in beer. Furthermore, the final pentanedione concentration in beer is strongly affected by an increase in isoleucine and valine wort levels, which leads to the reduction of pentanedione concentration in the fermented wort. Finally, none of the spectrum of wort nitrogenous compounds was found to significantly affect the formation of acetylaldehyde in fermented wort.

Additionally, the optimal nitrogen wort consistency was statistically calculated for the lager yeast strain SC3, with the intention that the wort fermentable sugars would be fully attenuated in the fastest possible time and also excellent beer quality be produced containing desired levels of total vicinal diketones and acetylaldehyde and many other flavor compounds. This statistically designed wort synergistic amino acid and ammonia combination can be used as the template for predicting the fermentation performance of other lager and ale yeast strains and also the quality and stability of the beer produced. Therefore, from this statistical nitrogen wort optimization, it became clear that for certain amino acids, it is essential that their relative proportions must be maintained in wort at standard values, whilst for other amino acids the relative concentrations are not significant apart from their contribution to the total assimilable nitrogen. However, Pugh *et al.* (1997), during their supplementation experiments, observed that when additional levels of amino acids were added to low FAN worts, the negative impact of low wort FAN levels on yeast growth was largely, but not completely reversed, by amino acid reconstitution. Such an effect suggests that wort nutrients other than FAN may also be affecting the rate of fermentation and for that reason the brewer should not only take the FAN malt and wort content under consideration before commencing a new fermentation.

5.4 Mashing experiments

The main conclusion that arises from conducting mashing experiments with 28 different types of barley malt samples is that a negligible proportion of yeast assimilable nitrogen is produced during this processing step. It was shown that 88-90% of wort free amino nitrogen is formed during barley grain germination and the remaining FAN is produced during mashing by the activation of mash proteases at 65^{o}C. The literature suggests that the FAN fraction in malt and the resulting mash is 70/30 (Lie, 1973; Taylor and Boyd, 1986) was unconvincing, since it was shown that a more realistic version of this ratio is 90/10. The FAN distribution 70/30 ratio seems to be more applicable when the total malt and wort amino acid fraction was analytically investigated. In more detail, it was found that 71% of free wort amino acids originates during barley germination, whilst the rest of the proportion of wort amino acids occurs during mashing by the activation of mash proteolytic enzymes. When the ammonia wort content was analyzed before and after mashing, it was found that 75% of ammonium ions was already formed in the malted grain prior to grist mashing at 65^{o}C.

However, even if the brewer has established, after having conducted a great number of fermentations, an appropriate wort amino acid configuration for a specific yeast strain, it is not feasible to interfere with a particular stage of the malting process to enhance or suppress the production of specific amino acids. This is because of the large number and complexity of relevant mechanisms making it difficult to elucidate for a certain amino acid concentration in malt, how much is generated by direct degradation of high molecular protein, how much is formed by transamination of other amino acids and how much is synthesized from carbohydrates or fatty acids or formed as by-products of the TCA cycle.

5.5 Overall conclusions

In general, fermentation results indicated that wort FAN correlates well with at least three fermentation performance indicators. Firstly, high initial FAN content allows a more efficient reduction of the wort gravity. Secondly, pH decrease during fermentation is proportional to the amount of FAN utilized and thirdly the FAN wort content is suggested to be a useful index towards the formation of total VDKs, esters and higher alcohols in the later stages of fermentation. Wort free amino nitrogen has a direct influence on beer quality, through its components and metabolites surviving in the final product to determine some key aspects of beer flavour, but also through its impact on yeast performance. It is suggested that

the most useful index of tolerance is the flavour compounds that display the most sensitive reaction to a change in one or more wort FAN compounds. Building on this information, there is an urgent need for the construction of models relating wort FAN composition and yeast quality to flavour compound production. Such a model will assist the brewer in decisions concerning how much effort is needed to control wort free amino nitrogen composition. Most brewers simply rely on the overall strength of the wort specific gravity as an index of fermentation quality and yeast quantity, assuming that the relative balance of nitrogenous materials remains constant. In terms of beer flavour, it is not only a matter of the initial wort FAN content, but equally the amino acid and ammonium ions equilibrium in the medium and a number of fermentation parameters.

To conclude, knowledge on the roles of nitrogenous components of malt and wort in order to meet yeast requirements has increased substantially. Nevertheless, optimization of the nitrogen content is a very complicated issue due to the large number of nitrogen compounds found in malt.

Chapter 6: Future Work

The experimental work presented in this thesis can be used as a reference point so that research regarding the free amino nitrogen content of malt and wort can be further investigated. One of the first areas for further investigation is to continue the supplementations of the same high gravity adjunct wort (15^{o}Plato + 30% VHM syrup) used for the conduction of all the supplementations with the same combinations of amino acids and ammonia concentrations, illustrated in tables **7A** and **7B**. However, this time the yeast strains such as **SC4** and **SC8** should be used in order to compare the findings with the results obtained from the **SC3** strain.

In addition, it is also suggested that a series of static fermentations be conducted with the malt varieties closest to our statistically analyzed ultimate amino acid and ammonia wort pattern, in order to confirm if this amino acid sequence is the optimal one for the yeast strain SC3.

Another series of experiments of interest would be fermentations, in which an artificial wort type containing all the required fermentable sugars, but only one di-peptide and or one tri-peptide, as the sole source of assimilable nitrogen is used. In addition to the yeast fermentation behaviour in the presence of only small peptides, its metabolic activity under extremely limiting nitrogen conditions could be examined. Simultaneously, the general protease activity and the rate of peptide degradation should be measured under these fermentation conditions.

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